THE USE OF "PURIFIED" RDE IN THE DESTRUCTION OF NON-SPECIFIC INHIBITOR IN SERUM

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In titrations of human sera for antibody capable of inhibiting haemagglutination by influenza virus, the occurrence, particularly with some strains, of non-specific inhibition is a well recognized source of difficulty. Much work has been undertaken with a view to the removal of the inhibitors concerned, notably by Mulder and van der Veen (1948) and van der Veen and Mulder (1950). Following work in this laboratory on the receptor destroying enzyme (RDE) of *Vibrio cholerae*, these authors recommended treatment of sera with crude filtrates of *V. cholerae* as the most satisfactory method. They noted, however, the lack of correspondence between the receptor destroying enzyme (RDE) activity of the filtrates and their ability to destroy non-specific inhibitor. Appleby and Stuart Harris (1950) and Chu, Andrewes and Gledhill (1950) also reported the failure of "purified" RDE to inactivate the inhibitor and attribute the success of the crude filtrate to the presence of another enzyme.

Isaacs and Bozzo (1951), however, have investigated the phenomenon from this point of view and conclude that RDE is the factor responsible. They have shown that if another enzyme is involved then it has remarkably similar properties to RDE. White (unpublished) has been able to destroy non-specific inhibitor in human serum with "purified" RDE but not that present in ferret serum.

In view of these conflicting reports it seemed desirable to investigate some of the possible reasons for the failure of purified RDE to give consistent results in the hands of different workers. The term "non-specific inhibitor" is used to describe inhibitor to active virus and "Francis inhibitor" for inhibitor to indicator (inactive) virus.

Materials and Methods.

Viruses.

The following strains were used in the form of infected allantoic fluid harvested from eggs inoculated at 11 days and incubated for 48 hours:

Influenza A. MEL (Melbourne).
CAM (A^1 strain known to give high non-specific titre).
Influenza B. LEE.

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Sera.

For early experiments random samples of human sera were obtained from normal individuals. The immune sera we obtained by courtesy of Dr. S. G. Anderson of this Institute and were from the Ocean Island influenza epidemic of 1951.

Three normal ferret sera were obtained and also two immune ferret sera after intranasal inoculation with MEL and LEE.

Calcium acetate saline. Isotonic sodium acetate-acetic acid buffer adjusted to pH 6·2 and containing 0·1 p.c. calcium chloride.

V. cholerae filtrates were prepared according to the method of Burnet and Stone (1947).

"Purified" RDE had been prepared by the method of Ada and French (1950).

Titrations of non-specific inhibitor.

Serial twofold dilutions of serum (0·25 ml.) were prepared in saline and an equal volume of the virus, diluted in saline to contain 5 agglutinating doses (AD), was added. Finally, 0·25 ml. of 1 p.c. washed fowl cells were added and allowed to settle at room temperature for thirty minutes. The end point was taken as 50 p.c. (+) agglutination.

RDE titration.

Both crude Vibrio filtrate and "purified" RDE were titrated and diluted in calcium acetate saline to contain 100 units RDE/ml. The method used was that of Burnet and Stone (1947).

EXPERIMENTAL.

Sera were diluted 1 in 5 (0·25 ml. serum in 1·0 ml. of calcium acetate saline containing 100 units RDE) and held at 37° C. for 18 hours. A drop of toluene was added to stop bacterial action. The RDE activity was then destroyed by heating to 62° C. for thirty minutes. Controls included untreated sera and sera heated to 62° C. for thirty minutes in calcium acetate saline.

TABLE 1.

Action of crude filtrate and "purified" RDE on normal human sera.

Serum No.	Treatment	Test viruses MEL	CAM	H.LEE
1	Untreated	<10	40	1,280
	Heated 62° C.	<10	30	3,840
	Treated { Crude filtrate	<10	<10	<10
	{ Purified RDE	<10	40	320
2	Untreated	10	80	2,560
	Heated 62° C.	<10	80	7,680
	Treated { Crude filtrate	<10	10	<10
	{ Purified RDE	<10	80	480
3	Unheated	<10	20	1,280
	Heated 62° C.	<10	20	5,120
	Treated { Crude filtrate	<10	<10	<10
	{ Purified RDE	<10	<10	<10
4	Untreated	<10	15	3,840
	Heated 62° C.	<10	15	5,120
	Treated { Crude filtrate	<10	<10	<10
	{ Purified RDE	<10	20	400

5 AD of MEL, CAM and H.LEE were used in titrations for antibody, non-specific and Francis inhibitors. Although the crude filtrate destroyed both inhibitors the "purified" RDE did not completely destroy even the Francis inhibitor. These results are shown in Table 1.

A possible explanation of these results lay in the consideration of pH and the buffering qualities of the different sera. The action of RDE is known to proceed at an optimum of pH 6·2 (Burnet and Stone, 1947). Using the same technique is outlined above, the action of "purified" RDE was studied over the range pH 4–8.

Table 2 shows that the action falls off rapidly at a pH above 7.

TABLE 2.

Destruction of inhibitor for H.LEE in normal serum by RDE at different pH's.

Control untreated	0·25 ml. serum treated with 100 units RDE for 18 hours at 37° C. at pH.				
	4	5	6	7	8
5,120	120	50	20	120	2,560

pH estimations were therefore carried out on the serum—calcium acetate saline—RDE mixture and in all cases were found to be above pH 7. A duplicated range of sera were then treated with purified RDE, one set with pH adjusted to 6·0 and the other set with no adjustment of pH. For the majority of sera one drop of N/5 HCl was required to bring the pH down to approximately 6. Titrations were then carried out for the presence of non-specific inhibitor using 5 AD of CAM. Complete destruction occurred at pH 6 but there was some residual inhibitor present in the majority of those sera which had no adjustment of pH. These results are shown in Table 3.

TABLE 3.

Destruction of non-specific inhibitor by "purified" RDE at pH6

	Serum No.	Untreated	Heated at 62° C. for 30 minutes	RDE treated with pH adjusted to 6·2	RDE treated with pH not adjusted
Human					
	7	30	30	<10	20
	8	30	30	<10	<10
	9	100	60	<10	10
	10	60	50	<10	20
	11	50	30	<10	20
	12	60	60	<10	30
Ferret					
	373	50	60	<10	20
	378	100	60	<10	40
	379	60	60	<10	10

Non-specific inhibitory titres using 5 AD CAM.

Further investigation showed that the action of RDE in crude filtrate is also dependent on pH.

Table 4 shows the inhibitor titres of serum after treatment with 100 units of RDE, in crude filtrate and also in purified form. pH was adjusted to 6·2 and 7·6.

TABLE 4.

Titres of non-specific and Francis inhibitors after action of RDE at pH 6·2 and 7·6.

	CAM	H. LEE
Control heated 62° C. for 30 minutes	50	5,120
Treated with crude filtrate at pH 6·2	<10	30
Treated with crude filtrate at pH 7·6	40	1,920
Treated with purified RDE at pH 6·2	<10	15
Treated with purified RDE at pH 7·6	30	320

Although it is known that RDE does not lower the titre of specific antibody to any appreciable extent it was thought advisable to check this fact at pH 6·0.

A number of immune sera showing a definite antibody titre to CAM were used. The results of these antibody titrations before and after RDE treatment are shown in Table 5. There is slight reduction in titre after RDE treatment but this is probably due to removal of the non-specific inhibitor.

TABLE 5.

Resistance of specific antibody to treatment of serum with RDE at optimal pH.

Serum No.	Untreated	Heated at 62° C. for 30 minutes at pH 6	Treated with RDE at pH 6
196	60	40	30
352	100	80	80
515	240	200	180
646	120	110	80
672	200	200	130
720	160	160	100
808	80	60	60
980	100	60	80

Two ferret sera were also available. The animals had previously been inoculated intranasally with MEL and LEE. The antibody titres before and after RDE treatment were 2,560/1,920 for MEL and 960/960 for LEE.

DISCUSSION.

Burnet and Stone (1947) have shown that pH plays an important part in the action of RDE. They showed the optimum to be pH 6·2. This fact does not seem to have been fully utilized by the majority of workers when attempting to remove non-specific inhibitor from serum and this probably accounts for the failure of purified RDE to give satisfactory results in the hands of some authors. These results show that both crude Vibrio filtrate and "purified" RDE when used in diluted form require a pH of approximately 6·0 to show optimal activity. The crude filtrate made by the method of Burnet and Stone is heavily buffered to pH 6·2 and when used, as by most workers, in undiluted state, remains at this pH after the addition of serum. The addition of serum to a solution which is not heavily buffered raised the pH to above 7·0 and if complete removal of the inhibitor is to be achieved the pH must be lowered to approximately 6·0.

Mulder and van der Veen (1950) have used crude filtrate at higher pH (up to 7·6). The high concentration of RDE in the reaction mixture, however, probably accounts for the successful removal of inhibitor. When RDE is used in low concentration, whether in purified form or in crude filtrate, an optimum pH is of vital importance.

During the preparation of "purified" RDE it is possible that slight structural modification of the molecule occurs with a resultant increased dependence on optimum pH. These results support the findings of Isaacs and Bozzo that RDE is the factor responsible for destruction of non-specific inhibitor in serum.

It was noticed in the course of experiments with H.LEE that heating of the serum to 62° C. increased, quite markedly in some cases, the titre of the Francis inhibitor. This also confirms the reports of Hirst (1948) and McCrea (1948).

SUMMARY.

The non-specific inhibitor of haemagglutination by influenza virus, in human and ferret serum, can be regularly removed by the action of "purified" RDE provided the pH of the reaction mixture is adjusted to 6·0.

"Purified" RDE has no significant destructive action on specific antibody under these conditions.

The conflicting results of previous authors are discussed in the light of these findings.

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A METHOD FOR RAPID RECORDING OF SYSTOLIC AND DIASTOLIC BLOOD PRESSURES

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The conventional method of taking the blood pressure in man is inadequate in following rapid changes such as occur immediately after strenuous exercise of short duration. It became desirable in the course of experiments to be reported later to record these changes. Accordingly a method was devised whereby repeated brachial blood pressure values could be obtained accurately and rapidly with the ordinary inflatable cuff, using orthodox auscultatory criteria. Previous methods for automatically recording blood pressure (Gilson, Goldberg and Slocum, 1941, and many others) use microphones for recording the arterial sounds but in our experience the artefacts due to adventitious sounds are much more frequent than when a trained observer listens to the sounds.

METHOD.

The method consists of a modification of the conventional sphygmomanometer. Using the orthodox auscultatory criteria, the systolic end point is taken as the point at which the first arterial sound can be heard on slowly deflating the cuff, which is in its usual position around the brachial artery. The diastolic end point is taken as the transition from loud Korotkow sounds to muffled faint sounds.

Systolic pressure determination.

In order to register the systolic pressure, the brachial artery is first completely occluded by means of a conventional sphygmomanometer cuff; air is then allowed to escape through an adjustable leak. The observer listens through a stethoscope strapped to the subject's arm, till he can hear the first arterial sound. On hearing this sound he immediately presses a valve which allows air to re-enter the system raising the pressure in the cuff by 10–15 mm. Hg. The artery is thus completely re-occluded and the above cycle then recommences. By using a manometer in parallel with the inflating tube, the blood pressure is recorded graphically on a kymograph. Fig. 1 shows a typical systolic pressure tracing obtained by this method; the height of the blood pressure is measured directly from the tracing using a perspex scale. By this method ten to twenty-five readings can be obtained each minute.

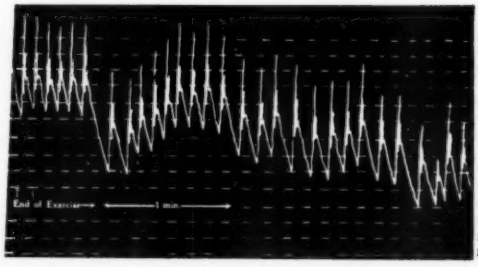

Fig. 1. Systolic pressure tracing taken during and after long term exercise. The height of the lowest point of each individual wave above the base line multiplied by two is the systolic pressure. Note recoil wave in each pulse resulting from the inertia of the mercury manometer.

Fig. 2 illustrates the details of the apparatus. The apparatus consists of a metal selector valve (A) which has two channels aa′ and bb′, either one of which may be open. In the figure the channels aa′ are open, air entering the system through (a), passing through the valve (dotted channel) and leaving the system through the other dotted channel and then through (a′). In order to open the valve to channels (b) and (b′) the left-hand side of the valve is pushed to the right when the dotted channels inside the valve will be opposite channels (b) and (b′). Channel aa′ is opened when recording the systolic pressure and bb′ is opened taking the diastolic pressure. The other feature of the apparatus is a metal hand-operated spring valve (B) which has two channels as shown, only one of which is open at a time. On depressing the valve for an instant the second channel is open and the first one closed.

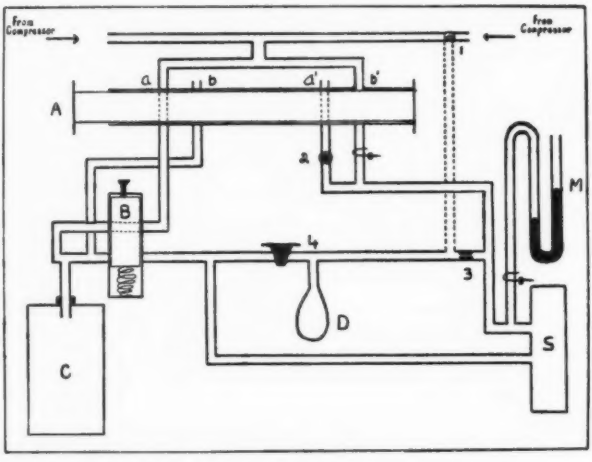

Fig. 2. Schema of blood pressure apparatus.

When the systolic pressure is recorded the apparatus is first filled completely in the air under pressure from an air compressor through tap (1). Air is then allowed to escape through an adjustable leak (2) so that the pressure in the circuit falls at a rate of 3–4 mm. Hg./second. Air in the meantime enters the reservoir (C) (a glass bottle of about 1 litre capacity), through (a) and the upper channel of valve (B) at a predetermined pressure from the air compressor (7–8 lb./sq. inch). When the operator hears the first Korotkow sound he depresses valve (B) for an instant. Air rushes from the reservoir (C) through the lower channel of valve (B) into the cuff (S) and raises the pressure above systolic. Some damping for this rush of air is provided by the bag (D) which is an ordinary rubber bag of about 2 litre capacity, and by a screw clamp on the mercury manometer lead.

Diastolic pressure determination.

In the ordinary clinical determination of diastolic pressure the end point is normally approached from above and is taken as the point at which loud arterial sounds suddenly become muffled and faint. For reasons to be considered later this procedure is undesirable for continuous registration of diastolic pressure and in the present method the diastolic end point is approached from below.

The cuff is first of all quite empty; air is then injected into the cuff, at first rather rapidly and then more slowly. When the end point is reached the cuff is immediately deflated and the cycle recommences. A typical tracing is shown in Fig. 3.

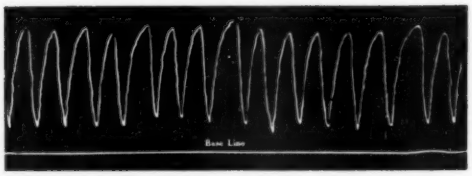

Fig. 3. Diastolic pressure tracing. The height of the peak of each wave above the baseline multiplied by two represents the diastolic pressure. Though the blood pressure cuff is always completely deflated between readings this is not shown in the tracing because of the damping of the manometer.

The details of operating the apparatus are as follows: The valve (A) is in the diastolic position with channels bb′ patent. Air enters the system through b′ from the compressor and goes into the cuff. Tap (4) is closed. When the diastolic end-point is reached the operator presses valve (B) and air rushes through channel (b) into the open. Very little air passes out of the balloon (D) because of the presence of a capillary tube of 1 mm. internal bore (3). When (B) is closed the cuff is re-inflated after 3–6 seconds and the cycle recommences. Five to ten readings may be obtained each minute.

DISCUSSION.

It is convenient to consider separately the relationship between the systolic and diastolic pressures respectively as measured by this method and the true values. We have not measured arterial pressures directly but Kotte, Iglauer and McGuire (1944) found good agreement between systolic pressures measured with the Hamilton arterial manometer and by the ordinary auscultatory method. In a series, covering a wide variety of circulatory states, the average discrepancy was less than 1 p.c. With diastolic pressures, however, no such good agreement was found and discrepancies of up to 20 mm. Hg. were noted. They concluded that whilst the auscultatory criteria for systolic pressures seemed valid, the same could not be said for the auscultatory diastolic pressure criteria. Wiggers (1947) supports the above conclusions.

Systolic pressure.

In a single measurement of systolic pressure the method described differs from the conventional one in that the pressure is measured with a damped and not an undamped manometer and the accuracy of recording depends on the promptness with which the observer depresses the valve on hearing the first arterial sound. When determinations were made with the present method alternately with an ordinary sphygmomanometer the results agreed to ± 5 mm. Hg. in each of a group of 20 subjects. Since the pressure falls continuously until the valve is depressed a considerable error might be introduced if the observer missed the first pulse sounds. When a second observer listened intently

through the same stethoscope by a T-piece attachment it was found that an inexperienced observer might miss 1-2 beats/minute, but with practice this source of error disappeared almost completely. Accuracy can only be achieved, however, if all distracting noises are excluded.

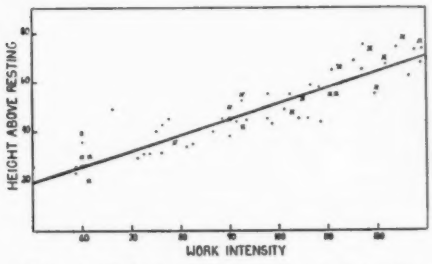

Fig. 4. Relation between work intensity (ft. lb./sec./10 stone) and the height of post-exercise blood pressure maximum (mm. Hg.) above resting level after 7 minute bicycle ride.

●Subjects with sphygmomanometer cuff empty during ride.

X Subjects with sphygmomanometer cuff inflated during ride.

In repeated measurements of systolic pressure the method differs from the conventional one since the cuff is inflated above the systolic pressure between measurements. It might be thought that, since the cuff was on for four to six minutes or longer at a time, the progressive element of discomfort might significantly affect the systolic pressure and thus greatly diminish the usefulness of the method. In practice, no discomfort was noted provided the arm was kept still. The principal discomfort occurs after the termination of an experiment when the cuff is released, when a sensation of pins and needles is generally felt. Moreover, statistical analysis of blood pressure tracings taken at rest does not show any progressive change in the means and variance of the systolic pressure level at various times from the start. Finally, Fig. 4 shows that the duration of inflation of the cuff did not affect the relation between the maximum blood pressure on cessation of exercise and the work intensity when the cuff is on during long periods of exercise. It can be seen that there is no systematic difference between subjects in whom the cuff was inflated 20 seconds before termination of exercise and those in whom the cuff was kept inflated throughout the exercise period (seven minutes). One may therefore conclude that provided the arm with the cuff is kept still, the cutting off of the circulation to the arm does not exert any general effects on the systolic pressure level.

Diastolic pressure.

The diastolic end point was approached from below for three main reasons; firstly in order to prevent the periphery from becoming engorged with blood, which might lead to fictitious pressure values; secondly to minimize the discomfort by leaving the cuff fully deflated between individual readings, and finally because during continuous registration the end point is less difficult to define, though even in this manner it is far harder to recognize than the systolic end point. The divergence between individual readings obtained by approaching

the end point from below and those obtained by approaching it from above is ±8 mm. Hg., which is approximately the same as the degree of divergence between individual readings of diastolic pressure. The diastolic readings obtained by this or any other auscultatory method are thus only useful in following fairly extensive changes in diastolic pressure. It is likely that the only really accurate diastolic readings are those obtained by the direct arterial puncture method.

SUMMARY.

A method for continuous rapid registration of systolic and diastolic pressures is described.

A brief discussion of the possible sources of error in the systolic and diastolic pressure determinations has been presented.

Acknowledgment. We wish to thank Mr. J. Stevens for his technical assistance in the building of the machine.

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THE NORMAL HUMAN BLOOD PRESSURE DURING AND AFTER EXERCISE, WITH SOME RELATED OBSERVATIONS ON CHANGES IN THE HEART RATE AND THE BLOOD FLOW IN THE LIMBS

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The main qualitative features of blood pressure changes during and after exercise in man have been studied on many occasions (Addis, 1922; Brittingham and White, 1922; Cotton, Rapport and Lewis, 1915; Eldahl, 1934; Gillespie, Gibson and Murray, 1926; Liljestrand and Stenstroem, 1919; Lowsley, 1911; Rapport, 1917, etc.). In general these consist of a rise in blood pressure during exercise; following exercise the systolic pressure is said to fall to within a few millimetres or even below the resting value (Cotton, Rapport and Lewis) and after that the blood pressure rises to a post-exercise plateau and then gradually declines.

Much less attention has been paid to individual variation and to quantitative features of the response, such as the relationship between blood pressure changes and the severity and duration of exercise, the type of exercise, etc. This applies particularly to the period immediately after cessation of exercise, when the rapidity of blood pressure changes poses considerable technical problems. Accordingly it was decided to take blood pressure readings at much shorter intervals than is customary during and after carefully graded exercises on a bicycle ergometer, in a fairly large series of subjects. This was made possible by use of a rapid method, described elsewhere (Cotton and Korner, 1952) of registering blood pressure.

To form an accurate picture of the vasomotor changes during and after exercise it is necessary to correlate measurements of heart rate, stroke volume, peripheral blood flow and limb volume made more or less simultaneously with the blood pressure readings. Little information is at present available concerning the period of rapid circulatory adjustment immediately following cessation of exercise. In particular, no observations seem to have been made immediately following exercise involving many groups of active muscles. It was therefore decided to study the heart rate, peripheral blood flow and limb volume in relation to the blood pressure changes, in the period immediately after exercise on the bicycle ergometer.

Methods.

The subjects used in these experiments were 21 medical students (15 men, 6 women), whose ages varied from 19 to 26 years. As the responses of the women did not differ from those of the men, all the results have been pooled.

All exercise was carried out on a standard bicycle ergometer and the work intensity was always expressed as foot pounds/second/10 stone body weight.

Systolic and diastolic blood pressures were recorded by means of a rapid recording device (Cotton and Korner). Heart rates were recorded by means of an electronic Waters Conley Cardiotachometer.

Qualitative changes in blood flow and volume in the extremities were recorded by means of an electric impedance plethysmograph. The apparatus used was based on a method developed by Atzler and Lehman (1932) in another connection. Details of the general principles of constructing the apparatus are given in Atzler and Lehman's paper and also by Nyboer (1944). The advantage of this particular type of plethysmograph over the Hewlett and Zwaluwenburg type is that it is completely inertia free and that the apparatus can be applied to the limb in a few seconds following exercise, thus making it particularly useful to follow the changes in blood flow and volume in the extremities, in the immediate post-exercise period.

RESULTS.

The blood pressure during and after sprint exercise.

All sprint exercises were of thirty seconds' duration, but were varied in respect to intensity, namely easy, medium and hard sprints. The work intensity varied from 60 ft. lb./sec./10 stone to 420 ft. lb./sec./10 stone (497 kg. m./min./64 kg. to 3,479 kg. m./min./64 kg.). Each subject carried out two rides of each of the three grades of sprint exercise.

On days when the systolic pressure was recorded, the subject (with cuff and stethoscope attached to his left arm) rested for ten minutes on the ergometer. A resting tracing of two to four minutes' duration was then taken. The thirty second sprint followed and in many experiments the systolic pressure was recorded during this period, but in some, the cuff pressure was merely kept elevated above the systolic point. The first post-exercise reading was obtained 1 to 8 seconds after exercise. Readings were then obtained at three to six second intervals for four to six minutes, depending on the grade of exercise.

When the diastolic pressure was recorded a procedure essentially similar to the above was followed. During the actual ride no readings were obtained owing to the difficulty in picking the diastolic end point amidst the incidental noises produced by the moving bicycle. The first post-exercise reading was usually obtained within five to ten seconds after exercise.

(a) *The systolic pressure.* Fig. 1 (A) is a graph of one of the commonest types of systolic pressure behaviour found during and after sprint exercise. It will be seen that the systolic pressure rises soon after the commencement of

the exercise and continues to rise steadily throughout the thirty seconds' sprint. At the end of exercise, the blood pressure falls to a post-exercise minimum (referred to as "trough" by Cotton, Rapport and Lewis), followed by a subsequent rise to a post-exercise maximum which is usually sustained as a plateau. Following this, the systolic pressure returns towards the resting value in a series of steps. This stepwise pattern has not been previously reported in the literature. Fig. 1 (A) shows the commonest pattern for the return of the blood pressure from the post-exercise plateau to the normal resting level. After 130 rides by 21 subjects this stepwise descent was observed in 109 cases. Only after 21 of the rides did the systolic pressure decline obliquely towards the resting level after the manner shown in Fig. 1 (B). When the subject assumed the supine posture following exercise an oblique descent of the systolic pressure level was noted in a significantly higher proportion of instances.

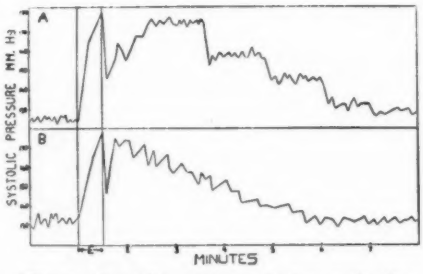

Fig. 1. Systolic pressure during and after thirty seconds heavy sprint. "E" denotes the period of exercise. The work intensity was 310 ft. lb./sec./10 stone in Fig. A and 300 ft. lb./sec./10 stone in Fig. B.

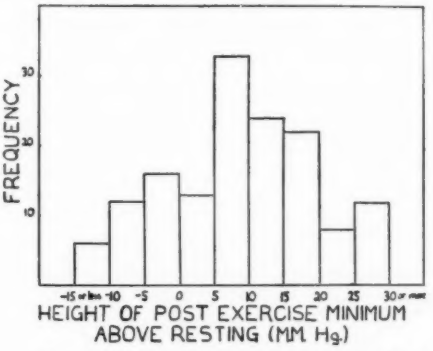

Fig. 2. Frequency distribution of height of post-exercise minimum ("trough") above resting.

While the systolic pressure fell immediately after cessation of exercise in all cases, the extent of the fall varied considerably. In Fig. 2 the minimum values obtained ("trough") have been plotted as a frequency histogram. It can be seen that the blood pressure fell to or below the resting level in only 40 p.c. of the observations. Cotton, Rapport and Lewis found that the blood pressure fell below normal in all their subjects. A much larger number of subjects was studied in the present investigation and statistical analysis of the data suggests that individual variation in response is responsible for the discrepancy. The first post-exercise reading was one to eight seconds after cessation of exercise in both Cotton *et al.* experiments and those reported here.

Immediately after the trough, the blood pressure rose abruptly to a fairly steady plateau level. The more severe the exercise the higher the plateau level

and the more slowly it was attained. The quantitative significance of these findings was assessed by deriving two regression equations:

$$H = 31 + 0{\cdot}095\ (W - 207) \quad . \quad . \quad . \quad . \quad . \quad (1)$$

where H is the height of the plateau pressure above resting,

W is the intensity of work in foot pounds/sec./10 stone.

The S.E. of regression coefficient is 0·011.

P for significance of regression coefficient from zero $<0{\cdot}001$.

In general terms, the equation indicates that for every 10 foot pounds/sec./10 stone increase in the intensity of work the post-exercise systolic pressure plateau is about 1 mm. higher.

$$T_p = 31 + 0{\cdot}134\ (W - 207) \quad . \quad . \quad (2)$$

where T_p is the time after exercise at which the plateau is reached,

W is the intensity of work in foot pounds/sec./10 stone.

The S.E. of the regression coefficient is 0·013.

P for significance of regression coefficient from zero is $<0{\cdot}001$.

The second equation indicates that the plateau is reached about 1·3 seconds later, for each increment in the intensity of work of 10 foot pounds/second/10 stone body weight.

In view of the fact that not enough time was available in the experiments to allow the systolic pressure to return to the resting level in every instance, a third regression equation has been derived, relating various factors to the time required for the systolic pressure to fall from the commencement of the plateau to within 20 mm. Hg. of the resting level. This represents on the average 40 p.c. of the total recovery time. As may be seen from Fig. 1, where recording was continued right to the return to the resting level, the recovery rate in the portion of the curve from the commencement of the plateau to within 20 mm. Hg. of the resting level, is roughly the same as that in the later portion of the recovery. Thus

$$T_r = 79 + 5{\cdot}17\ (H_{20} - 13) + 0{\cdot}12\ (W - 207) - 0{\cdot}02\ (T_p - 31) \quad . \quad . \quad . \quad . \quad (3)$$

where T_r is the recovery time in seconds as defined above,

H_{20} is the height of the plateau above resting minus 20 mm. Hg.,

T_p is the time after exercise at which plateau is reached,

W is the intensity of work done.

The first two regression coefficients are significantly different from zero.

The third regression coefficient is not significantly different from zero.

It appears then that the chief factor influencing recovery time is the height of the plateau; the significance of the term (W) of the equation indicates that, if the same plateau height is reached after different amounts of work, the case with the greater intensity of work will show a slightly slower rate of recovery.

(b) *The diastolic pressure.* The conclusions reached are based on medium and heavy sprints performed by each of the subjects. In view of the great difficulty of measuring diastolic pressures at rapid intervals by the auscultatory method (Cotton and Korner) the results are essentially qualitative in nature. In the present experiments it was not possible to record systolic and diastolic pressures simultaneously. The composite picture of changes in systolic and diastolic pressures has been reconstructed from the time relations of the data obtained and is shown in Fig. 5.

The systolic pressure during and after longer exercise.

Fig. 3 shows the systolic pressure behaviour during and after a bicycle ride of 110 ft. lb./sec./10 stone lasting ten minutes. There is a rise in blood pressure during the first three minutes of the ride and after this it is maintained at a constant plateau during exercise. After exercise there is sometimes a very slight post-exercise blood pressure trough but this is not nearly as constant a feature of the blood pressure behaviour as it is after a sprint. Following this the blood pressure rises to the usual blood pressure plateau and then it returns towards the resting level, usually in a series of steps but sometimes in an oblique fashion.

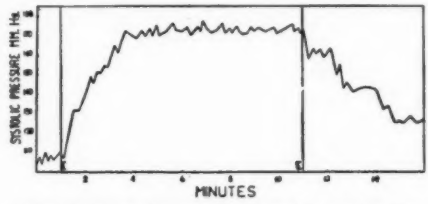

Fig. 3. Systolic pressure during and after long term exercise "E" is the period of exercise. The intensity of work was 110 ft. lb./sec./10 stone.

Statistical analysis of the results shows that the post-exercise blood pressure trough occurred in only five out of forty-eight cases (10 p.c.) following long term exercise, as contrasted with 83 p.c. of instances following sprint exercise. This difference is highly significant.

Analysis of the data also shows that the height of the post-exercise plateau above resting is related to the intensity of the work done. This is summarized in the regression equation:

$$H_L = 46 + 0{\cdot}609\ (W - 89) \quad . \quad . \quad . \quad . \quad (4)$$

where H_L is the height of the post-exercise plateau above resting,

W is the intensity of work in foot pounds/sec./10 stone.

The probability of the regression coefficient differing significantly from zero is $<0{\cdot}001$.

The equation indicates that, for an increment in the intensity of work of 10 foot pounds/sec./10 stone, the post-exercise plateau will be 6·09 mm. Hg. higher.

Finally, we have shown that the recovery time from the commencement of the plateau is related to the height of the plateau above the resting value and that an equation may be derived which is very similar to equation (3) to show this relationship. It appears that the time required for the systolic pressure to

fall from the commencement of the plateau to within twenty millimetres Hg. of the resting value is almost the same after long term exercise as after sprint, provided the height of the post-exercise plateau is similar in both cases. This occurs despite the great difference in the total amount of work done in the two instances.

The heart rate in relation to the blood pressure recovery after sprint exercise.

In 38 results obtained from 17 subjects heart rates were recorded simultaneously with the systolic blood pressure. The results from these experiments are summarized in Fig. 5. It may be seen that the heart rate recovery curve following sprint exercise may be divided into two stages. The first stage corresponds with the blood pressure trough and ends a little after the blood pressure has reached the post-exercise plateau. The recovery in the heart rate during this stage is fairly rapid, the heart rate dropping by about 20 to 40 beats per minute during the first forty to sixty seconds following exercise. In the second portion of the recovery there is a slower and more gradual fall in the heart rate. The heart rate recovery graph and the systolic pressure graph both show steps or oblique descents but there is no synchronisation between them.

Blood flow and volume changes in the extremities after sprint exercise.

The observations were limited to four subjects, each of whom performed three bicycle rides and in all experiments systolic (brachial) pressure was recorded in conjunction with the bloodflow and limb volume determinations.

(a) *Flow and volume changes in the calf.* As soon as the subject finished riding, he moved to the recording chair which was placed next to the bicycle ergometer, and records of bloodflow or limb volume, as well as of systolic pressure, were then taken. Blood flow readings were obtained at the rate of 3 readings per minute during the first two minutes of the post-exercise period and thereafter at the rate of two readings per minute.

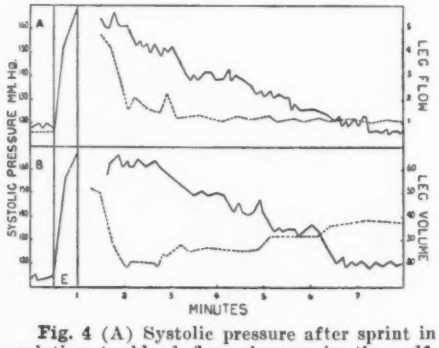

Fig. 4 (A) Systolic pressure after sprint in relation to blood flow changes in the calf. Blood flow is given only in relative units (tan x).

(B) Systolic pressure after sprint in relation to volume changes in the calf. Volume measured in arbitrary units.

———— Systolic pressure.
---------- Leg volume and leg flow.

A typical experiment is illustrated in Fig. 4 (A). The blood flow in the calf is high immediately following exercise during the period of the systolic pressure trough. The blood flow diminishes abruptly a little time after the

post-exercise plateau has been reached by the systolic pressure. After the abrupt decline in flow, the flow diminishes slowly and is still about twice the resting flow seven minutes after exercise.

TABLE 1.

Changes in blood flow and systolic pressure in immediate post-exercise period.

Subject	Rest flow tan x	Maximum post-exercise flow tan x	Flow at end of period of diminution tan x	Decrease in flow (p.c.)	Decrease in systolic pressure (p.c.)
B.M.	0·40	3·5	1·6	55	14
B.M.	0·60	3·8	1·4	59	14
B.M.	0·90	5·6	1·5	73	2
B.M.	0·55	3·8	1·6	60	12
G.K.	0·40	3·8	0·6	80	—3
G.K.	0·45	5·1	1·2	80	2
G.K.	0·45	3·4	0·9	71	5
J.W.	0·50	1·6	0·9	30	8
J.W.	0·45	1·3	0·5	62	4
K.S.	0·45	2·0	1·0	50	4
K.S.	0·80	3·5	1·3	65	2
K.S.	0·90	5·7	1·3	80	4

tan x. In the measurement of blood flow through the calf by the plethysmographic method, variations in flow are shown by variations in the angle $\times$ between the horizontal baseline and the record obtained after temporarily occluding the venous return.

Table 1 illustrates the extent of the rapid diminution in blood flow and compares it with the corresponding drop in blood pressure. There is thus a large diminution in blood flow, which sets in a little after the onset of the systolic pressure plateau. It cannot be accounted for by the relatively small changes in blood pressure which occur at the same time. One may thus deduce the occurrence of vasoconstriction in the leg vessels. After this vasoconstriction has taken place the flow in the leg vessels is about double the resting rate. The fact that, after this initial period of rapid decline in blood flow, the rate of flow fluctuates but little, does not imply the absence of further vasomotor adjustments in the leg. It must be borne in mind that a drop of about 30 p.c. in the mean arterial pressure occurs during the remainder of the recovery period. The fact that the flow remains relatively constant thus probably implies some vasodilatation in the active muscle following the initial vasoconstriction, *pari passu* with the blood pressure adjustments. That this is in fact what occurs may be seen in Fig. 4 (B), which graphs the changes in calf volume following exercise, in relation to the changes in systolic pressure. This curve is obtained without interfering in any way with the leg circulation, the leg being merely immobilized between the condenser plates of the apparatus. We note the sharp decrease in limb volume occurring at about the time of the systolic pressure plateau, and subsequent progressive increases in leg volume occurring *pari passu* with the decrease in the systolic pressure (i.e. vasodilatation).

Flow and volume changes in the forearm.

Fig. 5 shows that immediately after exercise there is a considerable decrease in forearm volume and flow, and a minimum value is reached about thirty seconds following exercise. Increase in volume and flow then occurs more or less abruptly, at a time roughly corresponding to the systolic pressure post-exercise plateau. The flow and volume of the forearm then show only slight further changes during the remainder of the post-exercise period.

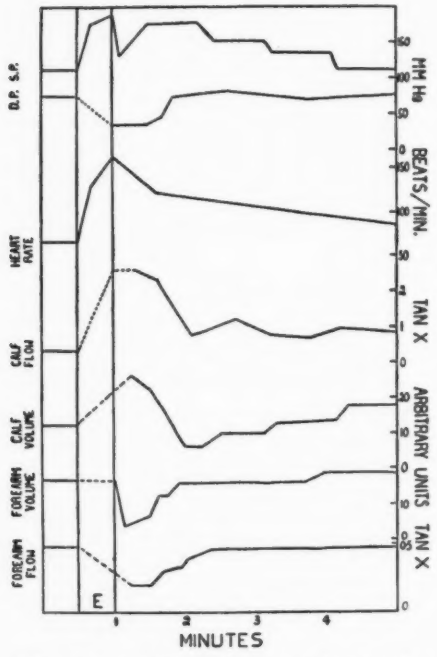

Fig. 5. Composite diagram showing trends and time relations of (1) Systolic pressure (mm. Hg.), (2) Diastolic pressure (mm. Hg.), (3) Heart rate (beats per minute), (4) Calf flow (tan x), (5) Calf volume (arbitrary units), (6) Forearm flow (tan x), (7) Forearm volume (arbitrary units). "E" is the period of exercise. Dotted lines show portions of curves for which no evidence is available.

DISCUSSION.

The systolic pressure after exercise falls to a post-exercise minimum ("trough"), then rises to a post-exercise plateau and then gradually recovers towards the resting level. The diastolic pressure on the other hand falls to a post-exercise minimum value and then returns to a level close to the resting value. It may thus be seen that the systolic pressure appears to reflect to a far greater extent changes which occur in the circulation following exercise, than does the diastolic pressure.

Fig. 5 represents a composite diagram showing the changes which occur in the various measurements which have been taken in this study following sprint exercise. It will be seen that the circulatory changes following sprint exercise may be subdivided into two phases.

During phase (1) the systolic pressure falls to a minimum post-exercise value ("trough"), and subsequently rises to a post-exercise plateau; the diastolic pressure falls to a post-exercise minimum value and later rises to a post-exercise plateau. The flow in the calf is elevated at the beginning of this

phase but towards the end of the period we have noted the onset of vasoconstriction in the calf. The forearm flow and volume are greatly diminished at the beginning of this phase but return to normal near the end of the phase.

During phase (2), the systolic pressure returns towards resting; the diastolic pressure is maintained at the level it reached at the end of phase (1). The blood flow in the calf is about double the resting flow and we have seen that there is progressive vasodilatation following the initial vasoconstriction at the end of phase (1), *pari passu* with the drop in blood pressure.

In the early stages of phase (1) the occurrence of the systolic and diastolic pressure troughs as well as the decrease in forearm flow and volume are strongly suggestive of pooling of blood. Cotton, Rapport and Lewis have suggested that this pooling occurs in the splanchnic area. From experiments to be published later, as well as from the calf flow and volume data, it would appear that a considerable degree of post-exercise pooling occurs in the previously active leg muscles following bicycle riding. At the end of phase (1) there is vasoconstriction in the previously active leg muscles as is seen in the decrease in leg flow and calf volume. These changes in the active muscles are probably designed to prevent the circulation from becoming depleted of blood due to pooling and to allow the blood pressure to become stabilized. During phase (2) the systolic pressure returns towards resting most commonly in a series of steps. The significance of the steps is not clear at the present moment. Together with the drop in blood pressure, there are vasodilator changes in the previously active leg muscles no doubt designed to maintain a high rate of blood flow despite the drop in the arterial pressure.

Following long term exercise the post-exercise trough is not nearly as prominent as it is after sprint. The most probable explanation would appear to be that the immediate post-exercise vasomotor adjustments are not as sudden as those following sprints owing to the more gradual application and lesser intensity of the stress of exercise.

It is of interest in connection with the blood flow results to note that most plethysmographic investigations of the effect of muscular exercise on blood flow in the past have involved exercises with large static components; they have comprised usually exercises involving small muscle groups only. In these studies (Grant, 1937; Grant and Pearson, 1937; Abramson, 1944 etc.), it was found that the blood flow was considerably increased at the end of exercise and then gradually declined. The discrepancy between the above work and the present results may be due to the fact that, in the experiments referred to, the exercise is too slight to produce any general effects and the resulting increase in flow thus represents the specific increase in response to local metabolic factors. In the present experiments general vascular reflexes are superimposed on these local factors.

SUMMARY.

The systolic pressure rises continuously during a thirty second sprint; following cessation of exercise it falls to a post-exercise minimum and then rises to a post-exercise plateau after which recovery occurs most commonly in a series of steps. The height of the plateau above resting is proportional to the work done.

The diastolic pressure falls to a minimum value following sprint exercise and then rises to a post-exercise plateau close to the resting value and remains there.

The flow in the previously active calf muscles is about seven times the resting value immediately after sprint exercise. At about the time of the systolic post-exercise plateau vasoconstriction occurs in the previously active muscles and the flow in the calf falls to about double the resting value. It is maintained at this level by means of vasodilatation of the leg vessels occuring *pari passu* with the blood pressure recovery.

The systolic blood pressure behaviour during and after long term exercise is described.

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REFLEX REGULATION OF POST-EXERCISE BLOOD PRESSURE

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A number of mechanisms have been suggested to explain the elevation of the blood pressure after cessation of exercise and its return to the resting level. Atzler (1931) and Rein (1931) suggested that metabolites from the muscles stimulated the vasomotor centre or the carotid and aortic bodies. Reflexes from the carotid sinus were considered by Christensen (1938) to restore the elevated blood pressure to normal resting values. Central vasomotor mechanisms linked with the performance of voluntary movement have been invoked (*cit.* McDowall, 1938).

Alam and Smirk (1937, a, b, c; 1938) on the other hand suggested that reflexes arising from the active muscles controlled blood pressure levels during and after exercise. Their conclusions were based on the results of experiments, in which the active muscles were rendered ischaemic during and after exercise. Only a very mild type of "static" exercise was employed in their experiments (6 foot pounds/second for 1 minute). In the present study it was decided therefore, to investigate the effects of occluding the circulation on the blood pressure changes after exercise involving many active muscle groups. Since occlusion of the circulation during exercise makes pressor effects of pain difficult to evaluate or control, attention was concentrated on recovery from exercise, rather than on the regulation of the blood pressure during exercise.

Methods.

Twenty medical students were used as subjects in the present experiments. All exercise was performed on a standard bicycle ergometer and was expressed as ft. lb./sec./10 stone body weight.

Systolic blood pressure was recorded by means of the rapid recording device described previously (Cotton and Korner, 1952). It has been pointed out elsewhere (Korner, 1952) that the systolic blood pressure reflects best the changes in mean arterial pressure which occur after exercise.

The circulation to the active limb muscles was occluded in most instances by rapidly inflating special rubber leggings, reaching about three-quarters of the way up the thigh to a pressure of 250 mm. Hg. Using an air compressor this pressure was reached in four to five seconds. The pressure rose to a height of about 150 mm. Hg., thus even then interfering markedly with the muscular blood flow, in one to two seconds.

In a few experiments ordinary leg cuffs were placed three-quarters of the way up the thigh. These were inflated to 250 mm. Hg. in less than one second. The results were similar to those of experiments using inflatable leggings, but the latter were far more comfortable. With leggings the pressure is fairly evenly applied over the whole limb surface and pain of the ischaemic type occurred in only two out of 100 experiments. A sensation of heaviness and pressure was usually experienced especially when the leggings were inflated immediately before cessation of exercise. The sensation of discomfort was maximal during the first half-minute and thereafter rapidly diminished.

Each subject attended for four experimental periods, with two weeks' interval between the individual experiments. In the first period the effect of the pressure leggings on the resting systolic pressure was first observed. After a rest period of fifteen minutes the subject carried out a thirty second near maximal sprint (about 310 ft. lb./sec./10 stone). At the end of the thirty second period the subject was stopped by rapidly inflating the leggings. Occlusion was maintained for four to six minutes (mean 5 minutes). Recording of the blood pressure was continued for some minutes following deflation.

In the second period the subject again carried out a near maximal sprint of thirty seconds' duration, but the leggings were inflated only after the post-exercise blood pressure plateau had been reached.

In the third period two medium thirty seconds' sprints were carried out. In the case of one of the rides the "first period" procedure was followed, whilst in the other the "second period" procedure was carried out.

In the fourth period after a near-maximal thirty second sprint leg cuffs were inflated to a pressure of 80-90 mm. Hg., thus interfering with the venous return without rendering the limb totally ischaemic.

RESULTS.

Effect of application of pressure leggings on blood pressure at rest.

A typical response is shown in Fig. 1. The blood pressure usually rises to a maximum value a few seconds after the application of pressure and then rapidly descends again and stabilizes itself at a lower level a little above the resting value.

The duration of application of pressure varied from two to three minutes. The mean height of the maximum was 14 mm. Hg. above resting and the lower plateau level was 6 mm. Hg. above resting and was reached 10-20 seconds following the maximum. Application of the pressure leggings in the resting non-exercised subject therefore results only in a slight and mostly transient increase in blood pressure.

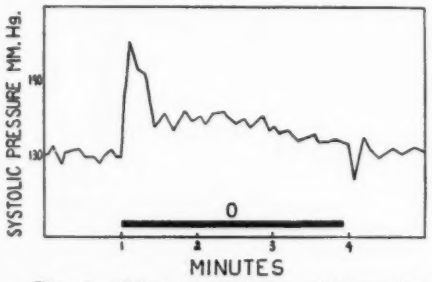

Fig. 1. Effect of occlusion of lower limb circulation on systolic pressure at rest. "O" is the period of occlusion.

Effect of application of pressure leggings at the end of thirty seconds' sprint.

To observe the effects on the entire course of blood pressure changes following exercise the pressure leggings were inflated at the point of cessation of exercise and the pressure was maintained for four to six minutes (mean 5 minutes). This time corresponds roughly to the time required for complete recovery in post-exercise blood pressure following a sprint of a similar intensity.

Typical results are shown in Fig. 2 and Fig. 3. In Fig. 2 the blood pressure after a heavy sprint ($W = 320$ ft. lb./sec./10 stone) is contrasted with a typical blood pressure response when the circulation is unoccluded. It can be seen that the blood pressure immediately after exercise ("trough") is higher with the circulation occluded, and that the post-exercise plateau is higher and is maintained.

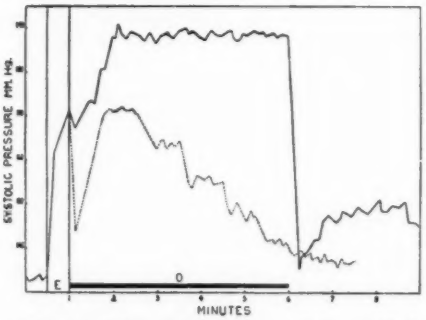

Fig. 2. Effect of occlusion of lower limb circulation immediately after heavy sprint on systolic pressure. "E" is the period of exercise, "O" is the period of occlusion.

——— Systolic pressure in occlusion experiment.

- - - - - - - - Systolic pressure in control experiment on same subject with circulation unoccluded.

Work intensity is 320 ft. lb./sec./10 stone.

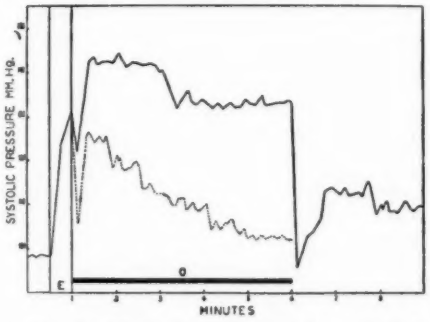

Fig. 3. Effect of occlusion of lower limb circulation immediately after medium sprint on systolic pressure.

——— Systolic pressure in occlusion experiment.

- - - - - - - - Systolic pressure in control experiment on same subject with circulation unoccluded.

Work intensity is 180 ft. lb./sec./10 stone.

Fig. 3 contrasts the response of two milder sprints ($W = 180$ ft. lb./sec./10 stone). Again the immediate post-exercise blood pressure ("trough") is higher with the circulation occluded. The post-exercise plateau is also somewhat higher and during the course of the occlusion there is some adaptation in the blood pressure, following mild sprints, as evidenced by the slight fall in blood pressure about two to three minutes after exercise.

The most important quantitative features of these results have been set out in Table 1.

TABLE 1.

Comparison of the effect of occluding the circulation to both lower limbs immediately after exercise with control experiments on same subjects in which the circulation was not occluded.

	Circulation occluded	Circulation free (Control)	Significance of difference
Mean height of trough above resting	22·2±1·4 mm. Hg.	8±0·7 mm. Hg.	P=<0·001 significant
Mean height of plateau above resting	47 ±1·5 mm. Hg.	34±0·8 mm. Hg.	P=<0·001 significant
Mean height of final blood pressure level during occlusion	39 ±1·5 mm. Hg.	34±0·8 mm. Hg.	P= 0·01 significant
Work intensity	240 ft. lb./sec./10 stone	240 ft. lb./sec./10 stone	
Number of experiments	38	144	
Mean height of plateau in subjects who show some b.p. recovery during occlusion	43 ±1·9 mm. Hg.	31±1·1 mm. Hg.	P=0·001
Mean height of final b.p. during occlusion in subjects who show b.p. recovery during occlusion	32 ±1·8 mm. Hg.	31±1·1 mm. Hg.	Not significant
Work intensity	208 ft. lb./sec./10 stone	208 ft. lb./sec./10 stone	
Number of experiments	18	144	

Occlusion, therefore, appears to eliminate partially the blood pressure drop immediately after exercise ("trough"), judged from the systolic pressures 6 seconds after exercise, which are compared in the first line of the Table. The difference is statistically significant. As the systolic pressure at the end of a sprint of specified severity is about 35 mm. Hg. above resting the average blood pressure drop with the circulation occluded was 13 mm. Hg. and with the circulation free 27 mm. Hg. The pressure suit thus reduces the trough by about half. It is thought that this effect is due to the prevention of pooling of blood in the exercised muscles. The drop that still occurs is probably due to pooling in other areas such as the unoccluded active muscles of the upper thigh.

The second and third lines of the Table show that the post-exercise plateau is higher when the circulation is occluded when compared with the control group, and that even the blood pressure level at the end of the occlusion period is higher than the post-exercise control plateau.

It has been seen that the only adaptation that occurs with the circulation occluded, occurs after milder sprints. Line 7 of Table 1 shows that, even in those subjects where some adaptation in blood pressure level took place, no real recovery from the effect of exercise, as judged by the equality of the blood pres-

sure levels at the final occlusion level and the unoccluded post-exercise plateau, has occurred. In the case of the harder sprints no appreciable recovery occurred during occlusion. It follows that the application of the pressure leggings greatly retards the blood pressure recovery following exercise.

As soon as the pressure leggings are deflated the blood pressure falls precipitously as is shown in Fig. 2 and Fig. 3. It is thought that this drop in pressure is due to extensive pooling of blood in the dilated vascular areas of the lower limb. After a short time the blood pressure rises to a plateau (mean height above resting $15 \pm 1{\cdot}1$ mm. Hg.). The height attained is directly proportional to the work done. Recovery from this plateau occurs in a series of steps (see Korner, 1952). The steps to plateau in this post-deflation period are much more prolonged than control values (mean post-deflation plateau duration 86 ± 5 seconds; mean control plateau duration $35 \pm 1{\cdot}9$ seconds).

Effect of occlusion of circulation to active muscles in the course of post-sprint blood pressure recovery.

To study the effects of occlusion of the circulation in the exercised muscles during the later stages of recovery, that is, after the post-exercise rise of blood pressure was well established, the pressure leggings were inflated 50-100 seconds after cessation of exercise and the occlusion continued for 4½–6 minutes (average 5 minutes). The results are based on 30 experiments carried out by twenty subjects.

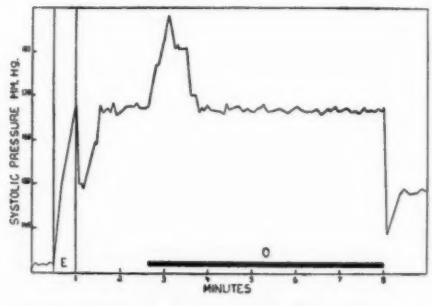

Fig. 4. Effect of occlusion of lower limb circulation 100 seconds after exercise on the systolic pressure. Work intensity is 290 ft. lb./sec./10 stone.

The changes in a typical experiment are shown in Fig. 4. In 22 out of 30 cases the blood pressure rose sharply, ("spike") as in Fig. 4, on occlusion of the circulation and then declined rapidly to a value near that before occlusion. In 8 out of 30 cases the sharp rise of blood pressure on occlusion did not occur and the blood pressure fell slightly for ½–1 minute again to a value slightly below that before occlusion. In 16 results there was no further adjustment of the blood pressure after the first "during occlusion" plateau had been reached; in 14 cases there was a further slight adjustment in the blood pressure usually in the form of a step. The results of this experiment are summarized in Table 2.

Line 1 of the Table shows that the blood pressure before occlusion was not responding in a significantly different manner in this experiment from control experiments on the same subjects. The fact that in over half the group the

TABLE 2.

Comparison of effect of occluding circulation of lower limbs in the course of post-exercise blood pressure recovery with control experiments on same subjects with the circulation free.

	Before occlusion	During occlusion	Control	Significance of difference
Plateau height above resting	37±1·6 mm. Hg.		34·6±0·8 mm. Hg.	P=0·2 not significant
Plateau height at first plateau during occlusion		32·6±1·84 mm. Hg.	34·6±0·8 mm. Hg.	P=0·4 not significant
Variance of plateau		3·4		
Final blood pressure height during occlusion		29 ±0·94 mm. Hg.		
Variance of final blood pressure height		0·89		
Expected blood pressure height in unoccluded circulation 77 seconds after exercise			31 mm. Hg.	
Work intensity		240 ft. lb./sec./10 stone	240 ft. lb./sec./10 stone	

blood pressure did not show any further adaptation once the first "during occlusion" plateau had occurred, whilst the remainder showed some further adjustment accounts for the difference in variance noted in lines 3 and 5 of the Table. The variance ratio is 3·8 and the probability of this occurring by chance is 0·001. The group as a whole behaves thus far more uniformly at the final level just before the release of pressure. This is thought to be due to the difference in speed of adaptation to the "mechanical" effects of the pressure leggings.

If we compare the final blood pressure height during occlusion with the expected control blood pressure level 77 seconds after exercise (the mean time of application of the pressure suit), it will be seen that the two values 29 mm. Hg. and 31 mm. Hg. do not differ significantly from each other.

It is clear that after five minutes' occlusion of the circulation to the legs the blood pressure level was still very close to the expected control level at the time of application of the pressure suit. In none of the experiments did the slight decline of blood pressure remotely resemble the normal process of recovery.

When the occluding pressure is released, the blood pressure falls abruptly as shown in Fig. 4. The fall is not as extensive or precipitous as in Figs. 2 and 3. After the initial drop the blood pressure becomes stabilized at a plateau level and the decline towards the resting value proceeds in steps. The plateau and steps are again longer sustained (mean 75 seconds) than the corresponding control values (mean 35 seconds).

Effect of partial ischaemia on the blood pressure recovery after sprint exercise.

The experiment consisted of 12 near-maximal thirty second sprints carried out by 12 subjects. Immediately at the end of exercise, thigh sphygmomanometer cuffs were inflated to a pressure of 80–90 mm. Hg. and this pressure was maintained for 4 minutes. At the end of that time the cuffs were deflated and recording continued for a few more minutes.

All the typical qualitative features of a sprint post-exercise curve were noted in this experiment. There was no significant difference in post-exercise plateau height above resting (mean 44 ± 3) in this experiment when compared with control values (mean 41 ± 2). The duration of the plateau and of the steps was significantly longer in this experiment (mean 77 ± 12 seconds) when compared with the control values (mean 50 ± 3). The probability for this difference occurring by chance is 0·03.

When the pressure in the cuff is finally lowered there is in all cases a small drop (10-20 mm. Hg.) and the blood pressure then continues to recover. This drop may be due to an increase in the velocity of the blood flow owing to removal of the obstruction from the leg. The experimental procedure thus prolongs the duration of the plateau but does not interfere with its height above the resting value.

DISCUSSION.

Under conditions of rest, the body is able to adjust itself fairly rapidly to the disturbances resulting from application of the pressure leggings. From those results it is clear that occlusion of the leg muscle circulation *per se* does not result in a sustained marked elevation of the blood pressure, the change being small and transient. This finding agrees with a similar experiment carried out by Asmussen and Nielssen (1938).

When the pressure leggings are applied immediately after exercise, mechanical disturbances resulting from this procedure (i.e. rapid increase in peripheral resistance) are to be expected. Thus the difference between control plateau height and plateau during occlusion immediately after exercise, and the sharp rise (''spike'') following occlusion some time after cessation of exercise may be in part accounted for by the mechanical action of the pressure suit. That the circulation is still capable of responding to the stress produced by occluding the lower limb circulation is especially clearly shown in the experiments where the circulation was occluded sometime after cessation of exercise. The disturbance represented by the ''spike'' is compensated almost as rapidly as in the ordinary resting experiments. The temporary depression of heart rate which we have observed during this ''spike,'' indicates that carotid sinus and allied reflexes are probably involved in the adjustments due to the mechanical overfilling of the circulation which results in the "spike.'' In the experiments where occlusion was applied immediately on cessation of exercise, the plateau during occlusion is both higher than the control level and is also longer sustained. In only the milder sprints was there any adaptation of the blood pressure level during the period of occlusion. We can say then, that mechanical effects are probably produced by the pressure leggings in all the experiments described. The disturbance is rapidly compensated when the pressure is applied either at rest, or a little time after the completion of exercise; whilst if the pressure is applied immediately after exercise the disturbance is compensated more slowly.

In all occlusion experiments there was a slight degree of adaptation of the blood pressure level in the course of an average period of five minutes' occlusion of the circulation to the lower limbs. As was pointed out under the relevant sections, the final occlusion level prior to deflation of the pressure leggings, was such that during the entire period of total ischaemia no real recovery in the blood pressure from the initial effect of exercise had occurred. Thus, if the active muscles are rendered ischaemic immediately after exercise, the final occlusion level even in the milder exercise groups where there is maximum adaptation in blood pressure is not significantly different from the control plateau. If the muscles are rendered ischaemic some time after the completion of exercise, the blood pressure level at the end of the five minute occlusion period is still at the level it would be expected to be at the time of occlusion.

Thus not only did occlusion of the circulation to the lower limbs maintain the blood pressure at an elevated level, but it maintained it very close to the expected level from the preceding effects of exercise. This fairly accurate correspondence between the blood pressure levels in the occlusion experiments and the control levels, suggests strongly that the blood pressure effect was the result of exercise and not of other factors such as pain.

The various current hypotheses of blood pressure regulation mechanisms after exercise have been detailed in the introduction. These various hypotheses will be examined seriatim in connection with the present findings.

If the elevation of the post-exercise blood pressure were to depend on direct central stimulation by muscle metabolites, then, since few metabolites are expressed by inflating the pressure leggings and almost certainly none by inflating the sphygmomanometer cuffs the post-exercise blood pressure should decline more rapidly after occlusion. As the blood pressure does not recover at all, one may conclude that the elevation of the blood pressure at the end of exercise is not due to central stimulation by metabolites.

The view that the carotid sinus and allied reflexes may be concerned with the blood pressure adjustments following activity has been summarized by Christensen. He thought that the muscles by local dilatation during and after exercise are responsible for their own increased blood supply and the pressor receptors compensate for this local shunt. According to this view the return of the blood pressure to normal would be due to a diminution in intensity of the pressor reflexes from the carotid sinus due to passive diminution in muscular blood flow following the end of exercise, as a result of the washing out of the local metabolites. As the pressure leggings would immediately remove this alleged local shunt, the blood pressure should recover more rapidly following occlusion if the above hypothesis were correct.

Finally, it is clear from the present experiments that exclusively central vasomotor mechanisms can be of no great consequence in post-exercise blood pressure regulation (McDowall, 1938), as occlusion of the legs should not have a very pronounced effect in retarding the blood pressure recovery in terms of this hypothesis.

In the light of the experiments described in this paper it is clear that the active muscles play an important part in the control of the blood pressure after sprint exercise. It has been pointed out that in none of the total occlusion experiments had there been any real recovery from the effects of exercise. The transient elevation of the blood pressure in response to application of the pressure leggings at rest and the "spike" reaction (Fig. 4) virtually exclude the possibility that the blood pressure may remain elevated merely for mechanical reasons.

As nerves constitute the only channel of communication between the legs and the rest of the body, it is therefore likely that these great delays in the

recovery of the blood pressure are due to afferent nerve impulses from the legs. It is thought that these afferent impulses may originate in the muscles in response to possibly some muscular metabolite. It is thought that as this potential metabolite is destroyed or reconverted the peripheral afferent stimuli diminish and the blood pressure then gradually declines.

Slowing the circulation through the limbs as in the partial ischaemia experiments, retards the blood pressure recovery presumably owing to the longer persistence of the peripheral afferent stimulus resulting from the slowed blood flow.

SUMMARY.

Evidence is presented for the existence of a reflex arising from previously active muscles, which is responsible for the maintenance and elevation of the blood pressure following sprint exercise.

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URIC ACID CONTENT OF KERATINOUS TISSUES AS DETERMINED BY THE URICASE METHOD

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In a preliminary communication (Bolliger and Hardy, 1944) it was reported that the clipped fur of the rabbit contains up to 500 mg. p.c.[2] of uric acid as determined by Folin's colorimetric method (1930). These then almost unbelievably high uric acid values in the fur of an animal whose other tissues are almost devoid of this compound (Folin, Berglund and Deric, 1924; Blauch and Koch, 1939) were confirmed by a few gravimetric estimations which showed an average uric acid content of over 400 mg. p.c. (Bolliger and Hardy, 1945).

Comparatively high uric acid values were also found in the keratinous structures of most other mammals, those in rabbit fur being the highest encountered. Furthermore, high uric acid values were observed in keratinous vertebrate tissue in general, such as birds' feathers, keratinous skin on birds' legs, claws, and shed snake skin (Bolliger and Gross, 1952). In these more recent investigations two colorimetric methods were used for uric acid determinations, the "direct" (Folin, 1930), and the "indirect" method of Folin (1934), the latter consisting in the isolation of uric acid as its silver salt prior to its determination. This "indirect" method usually gave lower values for uric acid than the "direct" method and sometimes the results obtained with the "indirect" method were only about half or less of those obtained with the "direct" method. This, for example, was the case with rabbit fur. It was, therefore, decided to employ a third method, which makes use of the conversion of uric acid to allantoin by the enzyme uricase, in order to clarify the uric acid values of keratinous tissue.

The shed skin of four snakes, the feathers of two birds and the fur of five species of mammals, including one marsupial, were extracted with boiling water as described previously (Bolliger and Hardy, 1945). A portion (5 ml.) of the sufficiently concentrated aqueous extract was used for uric acid determinations by Folin's "direct" method. A second portion

[1] Working under a grant from the National Health and Medical Research Council.

[2] For the sake of brevity, values per 100 gm. of dry tissue will be described as p.c.

was treated according to Buchanan, Block and Christman (1945). 5 ml. of the extract were incubated for 2 hours with a preparation of the enzyme uricase. After this the proteins of the uricase preparation were removed with tungstic acid and then Folin's "direct" method was applied to this incubated sample. The difference in colour value before and after treatment with uricase was considered to be a measure of the true uric acid content of the extract.

In addition the uric acid content of the original extract was determined by Folin's "indirect" method.

RESULTS AND DISCUSSION.

The results from the three methods for uric acid determination are presented in Table 1. Contrary to expectation (Bolliger and Gross, 1952) the values obtained by the uricase method are frequently closer to those obtained by Folin's "direct" method than those obtained by the "indirect" method. This is very marked in the case of rabbit fur, where the uricase method returns a value of only about 7 p.c. or less below that of the "direct" method, confirming the high uric content of rabbit fur and indicating that 400 mg. p.c. is approximately the lower level of uric acid. The fur of some breeds of rabbit contains as much as 780 mg. p.c. of uric acid by the uricase method. In most instances the values obtained by the uricase method are more than 50 p.c. higher than those given by the "indirect" method. Uric acid (10 mg. p.c.) added to rabbit fur extract in which most of the uric acid had been removed by acidification could be recovered completely within the limits of experimental error by the uricase method, but the indirect method recovered only 66 p.c.

Fur from the phalanger (*Trichosurus vulpecula*) is known to contain exceptionally high amounts of phenols which undoubtedly are responsible for fictitiously high uric acid values. This was supported by results with the uricase method. In one specimen of fur from the back of the phalanger, the uricase method returned only 46 mg. p.c., i.e. 56 p.c. of the uric acid as indicated by the "direct" method. The values yielded by the uricase method are even lower than those indicated by the "indirect" method.

Generally speaking, the "indirect" method was found to be unsatisfactory for the determination of uric acid in aqueous extracts of keratinous structures. This may be due partly to incomplete precipitation of the uric acid by silver nitrate and partly to the unpredictable chemical interference of the silver ion used as precipitant. In some instances, as for example in human hair, the "indirect" values were sometimes very much higher than those obtained with the "direct" method (Table 1).

The uricase method is said to be specific for uric acid, and we are thus compelled to accept the values which it yields even though they may appear extremely high as in rabbit fur and comparatively low in phalanger fur.

TABLE 1.

Comparison between "direct", uricase and "indirect" methods for the determination of uric acid in aqueous extracts of keratinous tissues.

	Uric Acid Method		
	"Direct" mg. p.c.	Uricase mg. p.c.	"Indirect" mg. p.c.
Shed Snake Skin.			
Japanese tree snake	409	390	303
Albino python	387	327	267
Black-head python	80	72	75
Water snake	206	160	76
Feathers.			
Rosella	152	138	85
Goose	171	152	108
Fur.			
Phalanger, dorsum	82	46	50
Rat	80	70	59
Guinea-pig	168	115	112
Rabbit, White	423	396	166
Grey	795	780	402
Black	782	770	517
Cat	270	264	160
Human hair.			
Female	9	4	16
Male	5	3	4
*Recovery**	109 p.c.	104 p.c.	66 p.c.

* 10 mg. p.c. uric acid added to rabbit fur extract after removal of practically all of the uric acid present originally.

However, an experimental error of ± 5 p.c. must be assumed to be inherent in the colorimetric technique employed.

SUMMARY.

In addition to Folin's "direct" and "indirect" colorimetric method, the uric acid content of a number of keratinous structures has been determined by its enzymatic degradation with uricase.

Though lower values were obtained by the uricase method they agree with the Folin's "direct" method frequently within 10 p.c. This is typified by the fur of the rabbit where the uricase and Folin's "direct" method agree closely. The "indirect" method, however, yields values only one-half or less of those shown by the preceding two methods.

The fur of the rabbit exhibited the highest uric acid content of all vertebrate keratinous structures examined.

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NITROGENOUS COMPOUNDS IN THE AQUEOUS EXTRACT OF VERTEBRATE KERATINOUS STRUCTURES

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The aqueous extracts of a number of keratinous structures of vertebrates have recently been analysed quantitatively for substances reducing before and after hydrolysis, ribose, desoxyribose, phenols and uric acid (Bolliger and Gross, 1952).

The present study deals with some quantitative aspects of the nitrogenous non-protein compounds which may be extracted from a variety of these keratinous tissues. Determinations of total nitrogen, ammonia nitrogen, urea nitrogen, "total" and preformed creatinine, creatine and uric acid were made on the fur of mammals, the feathers and keratinous skin of birds and the shed keratinous skin of snakes. For convenience the substances contained in the aqueous extracts of keratinous tissues are referred to as non-keratins.

METHODS.

The air dry keratinous substances were extracted with boiling water as described previously (Bolliger and Gross, 1952). The keratinous skin on birds' legs was detached from the underlying tissue and dried at 80° C. before extraction.

Total Nitrogen. Standard procedure according to Kjeldahl was adopted.

Ammonia Nitrogen. Aeration method according to Folin was employed.

Urea Plus Ammonia Nitrogen. The urease method combined with aeration (Van Slyke and Cullen, 1914) was employed. To obtain the urea nitrogen content the preformed ammonia, as established by separate analysis, was subtracted from the result of the urea plus ammonia determinations.

Preformed Creatinine. Shaffer's modification (1914) of Folin's method was employed. To avoid errors arising from partial conversion of creatine to creatinine during prolonged heating, in some instances one part of the keratinous substance was boiled for only one minute with about 10 to 15 parts of water in three successive extractions.

"Total" Creatinine. (Preformed creatinine plus creatine expressed as creatinine) and creatine (expressed as creatinine). Folin's method (1914) was used.

Uric Acid. 5 ml. each of the suitably concentrated aqueous solution was used for Folin's "direct" and the uricase method as described previously (Bolliger and Gross, 1952a).

In Tables 1, 3 and 4 "total" creatinine and uric acid are expressed as such, ammonia and urea by their nitrogen content.

[1] Working under a grant from the National Health and Medical Research Council.

RESULTS.

Keratinous Snake Skin.

As shown in Table 1, all shed snake skins examined contained considerable amounts of ammonia, "total" creatinine, and uric acid. The total nitrogen content of the aqueous extract of ten specimens of shed snake skin varied from 367 to 1,680 mg. per 100 gm. dry tissue[2] (average 714 mg. p.c.). The high average total nitrogen is mainly due to the two big values encountered in a tiger snake (1,350 mg. p.c.) and a water snake (1,680 mg. p.c.). In two out of three instances where dorsal and ventral skins were determined separately, values for the ventral skin were considerably lower than those for dorsal skin.

TABLE 1.

Observations on shed snake skins.

Snakes.	Total N mg. p.c.*	Ammonia N mg. p.c.	Urea N mg. p.c.	"Total" Creatinine mg. p.c.	Uric Acid "Direct" mg. p.c.	Uric Acid Uricase mg. p.c.
Boa-dorsal	522	34	—	118	111	—
-ventral	367	28	9	95	235	—
Boa-dorsal	340	33	0	70	148	—
-ventral	386	42	0	71	172	—
Diamond-dorsal	904	9	5	236	226	—
-ventral	571	30	0	200	196	—
Black	763	67	11	162	537	—
Tiger	1,350	25	0	530	315	—
Japanese Tree	460	20	3	88	409	390
Albino Jap. Tree	603	37	5	111	387	327
Blackhead Python	567	16	2	47	80	72
Water Snake	1,682	176	0	216	206	166
Undetermined	773	—	—	362	344	—

* In all the Tables this contraction stands for m.g. per 100 gm. dry tissue.

The ammonia nitrogen varied from 9 to 176 mg. p.c. The values for urea nitrogen varied from 0 to 11 mg. p.c., being zero in five specimens. In all the shed snake skins values for "total" creatinine were considerable, varying from 47 to 530 mg. p.c. A proportion of the "total" creatinine was preformed creatinine as indicated by qualitative tests. Only in five instances, where the rapid method of extraction was used, was the preformed creatinine determined quantitatively. It ranged from 16 to 47 p.c. of the "total" creatinine (average 30 p.c.), the remainder being creatine (Table 2).

Some of the uric acid values have already been published (Bolliger and Gross, 1952). On an average the nitrogen content of ammonia, urea, "total" creatinine, and uric acid amounted to only 196 mg. p.c. compared with a total nitrogen average of 714 mg. p.c., i.e. only 27 p.c. of the total nitrogen was accounted for.

[2] For the sake of brevity, values per 100 gm. dry tissue will be described as p.c.

TABLE 2.

Creatinine and creatine after short (3 × 1 min.) and some additional long (3 × 1 hour) boilings. The snake skin yielded approx. 100 p.c. of the extractable creatinine after short boiling.

Shed Snake Skin	"Total" Creatinine mg. p.c.	Preformed Creatinine mg. p.c.	Creatine mg. p.c.	Ratio Creatine to preformed Creatinine	
Tiger snake	339	55	284	5·2	Short extraction
Brown snake	171	41	130	3·2	Short extraction
Carpet snake	173	44	129	3·0	Short extraction
Black snake	183	65	118	1·8	Short extraction
Water moccasin	245	115	130	1·1	Short extraction
Feathers.					
Goose barbs	11	6	5	0·8	Short extraction
	8	5	3	0·6	Additional long extraction
Sparrow					
Total feather	15	9	9	0·6	Short extraction
	7	5	2	0·4	Additional long extraction
Pigeon.					
Total feather	7	7	0	0·0	Short extraction
	3	2	1	0·5	Additional long extraction
Chicken barbs	19	16	3	0·2	Short extraction
Duck.					
Total feather	18	18	0	0·0	Short extraction
Fur.					
Phalanger	22	7	15	2·1	Short extraction
Rat	38	17	21	1·2	Short extraction
	36	20	16	0·8	Additional long extraction
Guinea pig	11	9	2	0·2	Short extraction
	5	—			Additional long extraction
Cat	22	12	10	0·8	Short extraction
Rabbit	10	9	1	0·1	Short extraction

Keratinous structures of birds.

Total nitrogen, ammonia, urea, "total" creatinine, and uric acid were determined on the plumage of eight birds (Table 3). In four of these birds the larger feathers were dissected into barbs, shafts and barrels. Chemical determinations were then made on these three separate feather components.

Disregarding the comparatively low values obtained for the barrels, which represent only about one-fifth or less of the total feather weight, it is noteworthy that the values for total nitrogen of plumage show less variations than similar estimations on snake skins. The range nevertheless extends from 151 mg. p.c. to 383 mg. p.c. The ammonia nitrogen content of the feathers or parts of feathers varied from 19 to 206 mg. p.c. (average 65 mg. p.c.).

The urea nitrogen content was zero in all the feathers analysed.

"Total" creatinine was always present in the plumage (10 to 32 mg. p.c.) but using the slow method of extraction measurable amounts of creatine occurred in only 5 specimens of feathers examined. This was confirmed using the quick method of extraction which also showed that the "total" creatinine consists mainly of preformed creatinine, creatine being practically absent or present in small amounts only (Table 2).

Uric acid values given in Table 3 are similar to those found previously (Bolliger and Gross, 1952).

TABLE 3.

Observations on feathers and keratinous skin of birds.

Birds	Total N mg. p.c.	Ammonia N mg. p.c.	Urea N mg. p.c.	"Total" Creatinine mg. p.c.	Creatine	Uric Acid "Direct" mg. p.c.
Goose barbs	238	56	0	23	+	186
Goose shafts	308	46	0	27	+	116
Goose barrels	114	16	0	13	+	38
Goose kerat. skin	269	44	0	18	+	107
Duck barbs	239	71	0	32	0	117
Duck shafts	151	19	0	18	0	35
Duck barrels	89	19	0		0	28
Duck kerat. skin	195	32	0	17	+	
Pigeon barbs	348	99	0	18	0	199
Pigeon shafts	305	43	0	14	0	48
Pigeon barrels	216	22	0			31
Frogmouth small fth.	306	104	0	16	0	150
Frogmouth barbs	271	106	0	14	0	72
Frogmouth shafts	206	27	0			40
Gillbird total fth.	355	41	0	29	0	175
Bull Bull total fth.	383	74	0	25	+	135
Dove total fth.	367	94	0			145
Rosella total fth.	275	54	0	27	+	152
Bourke Parakeet total fth.				28	+	
Scarlet-chested Parakeet total fth.				21	+	

fth. = feathers.

The keratinous skin of the legs of birds presented a very similar picture to that observed in the plumage. Urea was also absent and the other constituents determined were present in amounts of the same order as in feathers.

Mammalian fur.

The hair of man, which is fundamentally different from the fur of mammals will not be referred to here, but some results are presented in Table 4.

The figures (Table 3) obtained for total nitrogen from fur (range 161 to 661 mg. p.c.) and ammonia (range 12 to 134 mg. p.c.), though somewhat higher, are comparable with those obtained from birds' feathers. However, in con-

trast to birds' feathers and shed snake skin urea is always present in the non-keratins of mammals.

TABLE 4.

Observations on mammals.

Mammals	Total N mg. p.c.	Ammonia N mg. p.c.	Urea N mg. p.c.	Creatinine mg. p.c.	Creatine	Uric Acid "Direct" mg. p.c.	Uric Acid Uricase mg. p.c.
Phalanger, dorsal fur	236*	33*	38*	33	+	82	46
ventral fur	412*	52*	91*				
Rat, fully grown	437	12	17	127	+	55	
Rat, ½ grown	288	18	17	64	+	80	70
Guinea-pig	532	134	74	26	+	168	115
Rabbit, black	661	55	51	31	+	782	770
Rabbit, white	520	40	52	19	+	450	
Cat	303	46	20		+	360	354
Dog	257	36	20	18	+	221	
Man♀	72	0·5	0·4	16	+	6	3
Man♂	90			11	0		

* Average of eight determinations on different animals.

On 8 different phalangers or opossums (*Trichosurus vulpecula*) total nitrogen, ammonia and urea nitrogen were determined separately on dorsal and ventral fur. The ventral fur on an average contained about twice as much of these nitrogenous compounds as the dorsal fur. The "total" creatinine values yielded by mammalian fur were, with the exception of the rat, approximately of the same order as those obtained from birds' feathers. The high "total" creatinine values of rats' fur resembled those of snake's skin. A large proportion of the "total" creatinine was preformed creatinine (Table 2).

The uric acid values include the high figures given by rabbit fur (Bolliger and Gross, 1952).

DISCUSSION.

Contamination.

Owing to possible contamination by urine, faeces, food, etc., analyses of keratinous tissues may be misleading. An attempt was therefore made to keep clean the keratinous structures on the animals to be examined. Healthy birds living singly or in small colonies in trees were seldom found to have contaminated feathers when shot down; but domestic birds, such as ducks or fowls kept closely confined frequently have dirty plumage unsuitable for analysis. The fur of mice, and particularly of rats, kept in colonies is heavily contaminated with dried urine, so that a total nitrogen content of nearly 2,000 mg. p.c. in the aqueous extract of the fur of such animals was not surprising. A fictitiously high uric acid content of rat fur (200 mg. p.c.), published previously (Bolliger, 1951), was in all probability largely due to contamination. Rats have, therefore, to be kept in individual cages and the fur

has to be washed with water several times a week or two before it is cut for analysis. Rinsing of the isolated keratinous integument with cold water may frequently remove contaminations without seriously affecting the non-keratin content of such structures.

In general, however, the findings were remarkably consistent and with the exception of very high figures observed in the shed skin of two snakes (Table 1) it was felt that the results may be accepted with a fair measure of confidence. The nitrogenous substances chosen for analysis (ammonia, urea, "total" and preformed creatinine, creatine and uric acid) were those which are mainly taken into account in vertebrate excretions.

The results for human beings as referred to in Table 4 will be discussed on another occasion because man with his relatively non-functional hair, low uricolytic index and peculiar grooming habits seems to be placed in a category of his own.

TABLE 5.

Average and range of nitrogenous constituents (expressed as nitrogen) in the aqueous extract of shed snake skin, birds' feathers and keratinous skin and mammalian fur.

	Total N. mg. p.c.		Ammonia N. mg. p.c.		Urea N mg. p.c.		"Total" Creatinine N mg. p.c.		Uric Acid N mg. p.c.		Average Determined N	Average Undetermined N
	Average	Range	Average	Range	Average	Range	Average	Range	Average	Range	mg. p.c.	mg. p.c.
Snake skin, 13 determinations on 9 species	714	340 to 1,680	43	7 to 145	3	0 to 11	64	10 to 144	86	23 to 179	196	518
Birds' feathers, 13 determinations on 8 species	274	151 to 383	65	19 to 106	0	0	8	4 to 10	40	13 to 66	123	151
Birds' keratinous skin, 2 determinations on 2 species	232	195 to 269	38	32 to 44	0	0	6		35		79	153
Mammalian fur, 23 determinations on 6 species	405	161 to 661	47	12 to 134	42	14 to 198	16	3 to 47	92	18 to 261	197	208

Ammonia and urea.

Every specimen of keratinous tissue examined contained ammonia. This is noteworthy because in the process of keratinization it does not seem to be necessary to convert most of the ammonia into other less toxic substances as it is done by the vertebrate body in its general metabolism. Avian keratinous integument on an average contains more ammonia than the other groups of keratinous structures examined (Table 5), but it is important that birds' feathers as well as the keratinous skin from birds' legs contain no urea. This is in marked contrast to mammals which always contain urea in their fur and

will be discussed under comparative biochemistry. About half of the shed snake skins examined also contained no urea, the remainder having only small amounts (2-11 mg. p.c. urea nitrogen).

Creatine and creatinine.

The separate determinations of creatine and preformed creatinine from the usual complete aqueous extract of keratinous tissues is not satisfactory, because in the process of repeated lengthy extractions with large volumes of water and their necessary subsequent concentration, about half of the creatine is converted into creatinine. Thus, the creatinine content becomes much higher than actually present in keratinous tissue. Consequently a rapid method of extraction has to be used to avoid the dehydration of creatine. For example, three boilings each of one minute duration of shed snake skin with small amounts of water, extracts practically all "total" creatinine but only a fraction of the uric acid. Such brief boiling gives an approximately true creatine/creatinine ratio as ascertained on solutions of known creatine/creatinine content.

It must be emphasized, however, that only about 65 p.c. of the creatine can be converted into creatinine, an equilibrium being reached on boiling in aqueous solution (Hahn and Barkan, 1920; Edgar and Shiver, 1925).

In conditions encountered in keratinous substances such as birds' feathers or certain mammalian furs, where the creatine concentration is very small and less than that of creatinine, apparently little dehydration of creatine or hydration of creatinine takes place even after prolonged boiling. However, the question of the reversible conversion of creatine$\rightleftharpoons$creatinine in small concentrations is not yet fully understood. Under these circumstances it is not considered advisable to present the creatine content of birds' feathers on a quantitative basis after prolonged boiling, and in Table 3 we have merely indicated the presence or absence of creatine. Furthermore, in contrast to shed snake skin rapid extraction of "total" creatinine from birds' feathers and certain mammalian furs is not complete (Table 2), and further investigations will be necessary to determine the optimum conditions for analysis.

The presence of considerable amounts of creatinine and often creatine in keratinous structures is somewhat unexpected because keratinization is not usually associated with muscle activity though admittedly the *arrector pili* is closely associated with the hair follicle, and in snakes powerful strands of muscle fibres occur beneath the dermis with fibres reaching up to the epidermis. The question therefore presents itself whether creatinine and creatine are derived from these skin muscles or is creatine involved in the energy reserve for the process of keratinization? This question cannot yet be answered.

However, analysis of the findings indicates that in terms of mammalian metabolism creatinine and creatine in keratinous tissues may be considered as

excretory products because the ratio of creatine to creatinine in such structures is comparable to that observed in the urine of higher vertebrates, that is to say, the amount of creatinine is comparatively large and frequently larger than that of creatine which is sometimes practically absent. In tissues other than keratinous ones, however, creatine is present in amounts about 30 to 100 times greater than creatinine. Thus the creatinine and creatine occurring in keratinous tissue afford yet another instance of the characteristically disproportionate distribution of non-keratins in this as compared with other tissues. This phenomenon is also seen with uric acid and pentose (Bolliger and Gross, 1952).

Uric acid.

The occurrence of large amounts of uric acid in the aqueous extract of keratinous substances has been referred to in previous papers (Bolliger and Gross, 1952 and 1952a) and the relationship to nucleic acid has been emphasized. In the present paper the so-called "direct" method of Folin has been used principally in the determinations of uric acid. The values for uric acid nitrogen are therefore in some instances too high. This, however, does not vitiate the findings of total determined nitrogen—it merely emphasizes the fact that the large figures for undetermined nitrogen are rather on the conservative side and possibly minimal. The difference between "direct" uric acid values and those obtained by the uricase method is particularly marked in some mammals as shown in Table 3.

Undetermined nitrogen.

Table 4, which summarizes the total findings on vertebrates, discloses that from 73 p.c. to 53 p.c. of the total nitrogen of the non-keratins is still undetermined. As already mentioned, some of this undetermined nitrogen may be accounted for as allantoin, amino acids, and purines. The results of assays for these constituents will be published on another occasion.

Some aspects of comparative biochemistry.

In spite of the fact that urea is present in appreciable amounts in birds' excreta, it is noteworthy that their feathers and the keratinous skin from their legs contain no urea whatever. This is in contrast to the fur of mammals which always contained urea (Tables 3 and 4). The presence or absence of urea in the non-keratins thus establishes a sharp dividing line between birds and mammals. A dividing line between the reptilian order *Sauria* and mammals is indicated by the absence of urea from shed snake skin or its presence in small amounts only.

According to their main nitrogen excretion product reptiles and birds are referred to as uricotelic animals in contrast to mammals which are ureotelic,

urea being the main excretion product. A sub-division of vertebrates into uricotelic and ureotelic according to their nitrogenous non-keratins is not feasible because all keratinous tissues examined contain considerable and frequently large amounts of uric acid, and even mammals with a high uricolytic index do not oxidize the uric acid in such tissues. However, the complete absence of urea in the non-keratins of birds provides an even sharper line of demarcation from mammals than that based on the frequently imperfect uricotelism observed in the excretions.

The "total" creatinine content seems to vary according to the class of vertebrates from which it originates. In keratinous snake skin, "total" creatinine is present in large amounts and the average "total" creatinine nitrogen is higher than the combined ammonia and urea nitrogens. In birds' feathers and keratinous skin from the legs the average "total" creatinine nitrogen is about one-eighth of that of ammonia. In mammals the "total" creatinine nitrogen amounts to about one-sixth of the ammonia and urea nitrogen. Thus, "total" creatinine is present in snake skin in outstandingly large amounts and approximately two-thirds of it is in the form of creatine. In contrast to this, creatine has frequently been found to be practically absent from birds' plumage or present in comparatively small amounts only. Thus, a dividing line between the reptilian *Sauria* and *Aves* is discernible with regard to the "total" creatinine and creatine content of keratinous tissue. Mammalian fur always contains preformed creatinine and creatine though with the exception of the rat definitely less than snake skin (Table 5).

The nitrogenous constituents of the non-keratins are subject to definite laws of comparative biochemistry which may be compared with those found for vertebrate excretory products. They refer directly to the mechanism of protein (keratin) synthesis in the epidermis without the intermediary action of organs of excretion. Whether these observations have any bearing on Needham's ingenious subdivision of the animal kingdom into ammono-ureo-and uricotelic groups remains to be seen.

The analytical findings for the five nitrogenous compounds determined and also the total nitrogen content of the aqueous extract of keratinous substances confirm the contention expressed previously (Bolliger and Gross, 1952) that the occurrence of non-keratins is not an isolated phenomenon observed only in certain species and partly or wholly due to contamination, but is common to the vertebrate integument in general. Not all of the non-keratinous compounds occur in every species examined, the absence of certain constituents indicating interesting relationships between the vertebrate classes, as for example, the absence of urea in birds' feathers.

A tentative interpretation of one aspect of the results would be to regard the periodic or continuous shedding of keratinous structures as a type of excretory process for the skin, by which non-keratins incorporated in the shed keratinous structures are eliminated.

SUMMARY.

Total nitrogen, ammonia, urea, creatinine, creatine and uric acid were determined on a considerable number of aqueous extracts of keratinous structures derived from shed skin of snakes, birds' feathers, birds' keratinous skin and mammalian fur and hair.

Ammonia was present in all vertebrate keratinous structures examined.

Urea was found consistently and in considerable quantities only in mammalian fur. Birds' feathers and the keratinous skin of birds' legs contained no urea and of the 11 shed snake skins examined, five had no urea and the remainder small quantities only. Thus by virtue of its urea content, mammalian fur is sharply demarcated from the feathers of birds, etc., and to a lesser extent from the keratinous skin of snakes.

"Total" creatinine was found in all the keratinous structures studied. The highest amounts, often well over 100 mg. per 100 gm. dry tissue, were encountered in shed snake skin, and the lowest in birds' plumage.

Birds' feathers also contain little or no creatine, which demarcates them further from the shed skin of snakes, these being rich in creatine. The sum of nitrogen estimated in ammonia, urea, "total" creatinine and uric acid accounts for only half or frequently less of the total nitrogen found in the aqueous extract of keratinous tissues.

The composition of the undetermined nitrogen fractions remains to be investigated for the full elucidation of the origin, nature, and possible function of the non-keratin components of keratinous structures.

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OBSERVATIONS ON THE METABOLISM OF MELANOPHORE-EXPANDING PITUITARY ("B") HORMONE AND ON THE EFFECT OF THIS HORMONE ON CARBOHYDRATE METABOLISM IN MAMMALS

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Current information on the properties of "B"-containing extracts is summarized in Waring and Landgrebe (1949, 1950) and Landgrebe, Munday and Waring (1950). The biological assay of "B" is described in Landgrebe and Waring (1950).

The recent interest in, and controversy around, the similarity and/or identity of ACTH and "B" (Sulman, 1952; Johnsson and Hogberg, 1952; Geschwind *et al.*, 1952; and Morris, 1952) demands *inter alia* a precise comparison of the properties of the two. This paper describes previously unrecorded information on the properties of "B"-containing extracts.

MATERIALS AND METHODS.

(a) "B"-containing extracts were made with dilute acetic acid from dried posterior lobe powder and were of two kinds: (i) Simple extracts filtered and sealed in tubes until required. This extract is subsequently referred to as "untreated." Such extracts have "B_1" pressor, antidiuretic and oxytocic properties; (ii) Simple extracts as above neutralized and then adjusted to N/10 NaOH, placed in a boiling water bath for 2 minutes, cooled, readjusted to pH 3·5, brought to the boil and sealed in tubes (Landgrebe, Munday and Waring, 1950). This extract is subsequently referred to as "treated." Caustic treatment destroys all the aforementioned activities except "B"; it doubles the strength of "B" and prolongs its effect when measured on *Xenopus*.

(b) Assays for "B" activity were made by dorsal sac injections into *Xenopus*. By definition an extract of 0·5 mg. International Standard Powder contains 1 International unit of pressor, antidiuretic and oxytocic activity. We have suggested (Landgrebe and Waring, 1944), that it also be recognized as containing one unit of "B" or melanophore expanding activity; this has not yet received official sanction so with regard to "B" activity we shall refer to "International Units" (I.U.).

(c) Blood sugar estimations were made by the Landgrebe, Munday modification (1951) of the Folin and Malmros procedure.

(d) Urines were all early morning samples. "B" activity was adsorbed with activated charcoal, eluted with acetic acid and precipitated with ether (Landgrebe and Waring, 1941,

1943). The precipitate was dried in a current of air and dissolved in Xenopus Ringer at pH 7·0 and injected into intact *Xenopus*. Extracts which evoked melanophore expansion were checked by injection into hypophysectomized toads.

EXPERIMENTAL.

Observations on the metabolism of "B"-containing extracts in mammals.

Estimation of "B" in urine. 100 ml., or more, of urine samples from 61 human subjects were examined for melanophore activity. Six of these subjects were Asiatics with moderately heavy skin pigmentation. A number of these urines yielded precipitates which evoked melanophore expansion in intact toads. None of these, however, caused any melanophore expansion in hypophysectomized animals. We can only conclude that our extracts contain pituitary stimulating substances but no "B" hormone itself. Similar results were obtained with sixteen samples of sheep urine tested in the same manner. These results confirm our previous finding on a more limited series and show further that with the extraction procedures used, which achieve considerable concentration, no "B" is detectable in the urine of moderately melanic humans.

Inactivation of "B" by animal tissue. Landgrebe *et al.* (1943) showed that under certain conditions a melanophore excitant substance could be recovered from macerated boiled, rabbit liver. Since then we have accumulated evidence that "B" is inactivated by liver, and that its destruction is enzymatic.

In one series of experiments 5 I.U. of "untreated" "B" were added to one guinea-pig liver homogenized in Ringer solution and incubated at 37° C. Portions of this mixture were withdrawn at intervals, filtered, and injected into intact *Xenopus*. Using a dose sufficient to evoke an m.i. of 4·5 at the beginning of the experiment, after 15 minutes the same dose raised the index to 3·0, and after 30 minutes no response was evoked. The same results were obtained if a small piece (2 gm. wet weight) of guinea-pig liver was placed in 100 ml. Ringer containing 5 I.U. of "untreated" extract.

The possibility that the disappearance of the hormone from solution was due to simple adsorption on the surface of the liver was studied by keeping another piece of the liver (2 gm. wet weight) in the refrigerator for a week without desiccation (until the enzyme activity was judged to be nil) and repeating the experiment. Samples removed and injected at various times up to 1 hour showed no measurable falling off in potency.

A cell-free extract of 2 gm. wet weight of guinea-pig liver (i.e. liver homogenized with Ringer and freed from cells by centrifuging at 3,500 revs./min. for 20 minutes) was made up to 100 ml. with Ringer and 5 I.U. of "untreated"

"B" hormone added. An equal quantity of "B" hormone was added to 100 ml. plain Ringer and the two solutions incubated at 37° C. in a water bath. The control solution showed no change in activity over this time period; that of the experimental solution declined steadily for 60 minutes. Cell-free extracts of boiled guinea-pig liver did not show any such effect after 2 hours of incubation with "B" under the same conditions.

Observations of the effect of "B" on adrenaline and burn hyperglycaemia.

In 1943 we reported that L.R.W.$_1$, a "B"-containing extract prepared by caustic treatment of an acetic acid extract and subsequent adsorption to and elution from charcoal, inhibited the hyperglycaemic response to adrenaline. In the experiments then reported the effect of 0·1 mg. of adrenaline was inhibited by 0·25 mg. (42·5 I.U.) of extract. In 1950 we reported inhibition of response to adrenaline by equal weights of "B"-containing extracts. These extracts had no measurable pressor or oxytocic properties and they were prepared from posterior lobe powder, not whole gland. Since an effect of adrenaline, a pure non-protein excitant, can be inhibited by similar weights of an admittedly impure protein extract, there is a strong temptation to believe that the inhibitory action is due to "B" itself: there is proof neither of this nor of the cognate proposition that a "B"-adrenaline antagonism exists as a normal physiological mechanism.

Burnt animals exhibit a hyperglycaemia in which the adrenal is involved (Clark and Rossiter, 1944) and in 1943 we made a few unrecorded experiments on the effect of "B"-containing extracts on this hyperglycaemia. This hyperglycaemia was also inhibited. Recently we have had the opportunity of repeating these experiments.

Extracts were adjusted to contain 20 I.U. per ml. Two injections of "B"-containing extracts were made subcutaneously into each animal, one 45 minutes before, the other immediately before injection of adrenaline or burning. Control animals received an equal bulk of saline at these times. Rabbits were clipped, anaesthetized with ether and dipped into hot water for the appropriate time. Blood was drawn from ear veins for sugar estimation.

Effect of "B"-containing extracts alone. Previously (1943) we found no consistent effect from the injection of a fairly pure "B"-containing extract in large doses, but we found some elevation from injection of cruder extracts which could have been due to inert protein or pressor or oxytocic activity. We confirmed in this set of experiments that simple extracts evoked a rise of blood sugar of the order of 40 mg. p.c. maintained for two or three hours and this could not be matched by injections of saline. To get the aforementioned elevation, 2 ml. of untreated extract (total of 40 I.U.) at 45 minutes' interval

were needed. We duplicated these experiments with the same rabbits and with the same extract inactivated by tryptic digestion. No elevation of blood sugar occurred.

Effect of "B"-containing extracts on adrenaline hyperglycaemia. In these experiments 0·1 mg. adrenaline was injected subcutaneously: untreated and caustic treated "B"-containing extracts were injected in divided doses totalling 40 I.U. These experiments confirmed our previous finding that adrenaline hyperglycaemia is inhibited.

Effect of "B"-containing extracts on burn hyperglycaemia. Clark and Rossiter (1943) found that the rise of blood sugar level after burning approximately one-third of the body surface at 70° C. for 30 seconds was similar to that observed after adrenaline injection of 0·3 mg./kg. Preliminary experiments of our own were sufficient to show that the hyperglycaemia from such treatment was inhibited by a total of 40 I.U. caustic treated extract. In two further sets of experiments the burning period was intensified to 40 seconds at 80° C. and the dose of "B" increased to 80 I.U. The controls showed the expected elevation of blood sugar. The animals receiving caustic treated pituitary injections exhibited a variable response. After the two injections, and the necessary preliminary handling, they all had blood sugars of about 140 mg. p.c. After burning one showed a slow steady rise of blood sugar so that after 5 hours it had reached 300 mg. p.c., a figure attained by controls 2½ hours after burning. Another maintained a level of 150-170 mg. p.c. for four hours and then exhibited a sharp elevation to the control level. The remaining two exhibited a level of 140 mg. p.c. for the 6 hours' duration of the experiment. Clearly further analysis is needed but the general conclusion is justified that "B"-containing extracts inhibit, in some measure, burn hyperglycaemia as well as adrenaline hyperglycaemia.

SUMMARY.

Examination of a more extensive series of urines, including some from Asiatics with moderately heavy skin pigmentation, confirms our previous inability to detect melanophore expanding ("B") hormone in them.

Liver and cell free extracts of liver inactivate "B" hormone *in vitro*. Boiled extracts of liver are without measurable effect on the hormone.

Injections of "B"-containing extracts inhibit adrenaline hyperglycaemia; they also inhibit, in some measure, hyperglycaemia resulting from controlled burns.

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THE EFFECT OF TEMPERATURE CHANGE ON ACCOMMODATION IN TOAD NERVE

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A series of measurements of the time constant of accommodation in nerve has been made, based on the theory of A. V. Hill (1936).

Experimental work by Solandt (1936), Schriever (1932) and von Kries (1884) indicates that accommodation in frog nerve decreases with fall in temperature, i.e. λ, the time constant of accommodation in milliseconds (which is used as the index of accommodation), increases with decrease in temperature.

According to Solandt (1936), "lowering the temperature of normal nerves by 15° C. (from approximately 20° C. to 5° C.) raised λ by 100 p.c. (8 determinations)." Solandt qualifies his conclusion by remarking that "the quantitative validity of these experiments is questionable as changes in temperature were quite slow and, owing to alteration of the preparation with time, a useful reverse in respect of temperature could not be obtained."

Schriever's (1932) experiments on change of Einschleichzeit with temperature are subject to similar criticism. He quotes one series only of λ determinations, which were apparently carried out on a single nerve-muscle preparation, and no control series of determinations was made.

To determine the effect of temperature change on accommodation, experiments initially carried out were such that the temperature of the whole preparation (muscle, nerve and neuromuscular junction) was altered. This was the method used by Solandt who, in a personal communication, states that "at lower temperatures experiments were carried out using a small gas refrigerator to hold the entire operation chamber and electrode assembly, and provided with a glass door for observation."

In preliminary experiments it was found that lowering the temperature of the whole preparation produced no consistent alteration in accommodation and produced an elevation in the initial threshold of the nerve to a rectangular pulse.

It was decided that a method should be used whereby the temperature of the nerve alone could be altered, that of the neuromuscular junction and muscle being maintained at room temperature.

Method.

Mathematical determination of the time constant of accommodation (λ milliseconds).

The method used is that described by Solandt (1936), the details of which will not be repeated here.

It was found that λ could be determined accurately using a condenser range from 0·1μ F up to 0·6μ F, instead of up to 1·0μ F as in Solandt's method. The advantage of using the shorter range was that it reduced the time over which readings were made.

Experimental material.

The sciatic-gastrocnemius preparation of the Queensland toad (*Bufo marinus*) was used.

The preparation was made according to the usual technique, and the index of excitation was the muscle twitch as observed by the eye.

Ringer's solution, used for soaking the preparations, and also as a saline bridge in the calomel half-cells, had the following composition:

NaCl 0·65 gm.; KCl 0·014 gm.; $CaCl_2$ 0·012 gm.

$NaHCO_3$ 0·02 gm.; NaH_2PO_4 0·001 gm.; per 100 ml. of water.

A fresh solution was prepared each day.

Means whereby temperature change was produced and controlled.

A small glass chamber saturated with water vapour was used as the test chamber for the nerve. This chamber was fitted into the side of a water bath, the temperature of which could be altered and maintained as desired.

The temperature of the nerve whilst in the test chamber was accurately determined by thermocouple.

The apparatus was so designed that the muscle and neuromuscular junction were outside the test chamber and thus remained at room temperature throughout the experiment (Fig. 1).

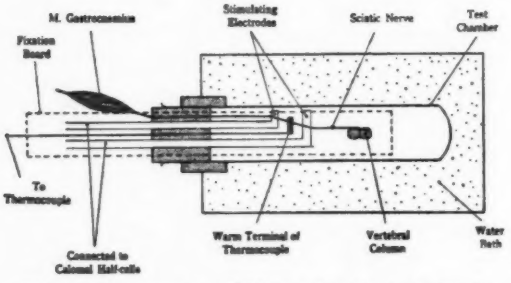

Fig. 1. For purposes of illustration the test chamber is much larger in proportion to the water bath, than was actually the case.

Conditions of experimentation.

Experiments were made on 32 days between August and November, 1947, with a fluctuation of room temperature from 12-28° C.

There were no means of controlling room temperature, and this was thought to be unnecessary, as the values of λ found at various room temperatures could be used in a distribution curve to determine the general trend in accommodation over a wide range of temperatures.

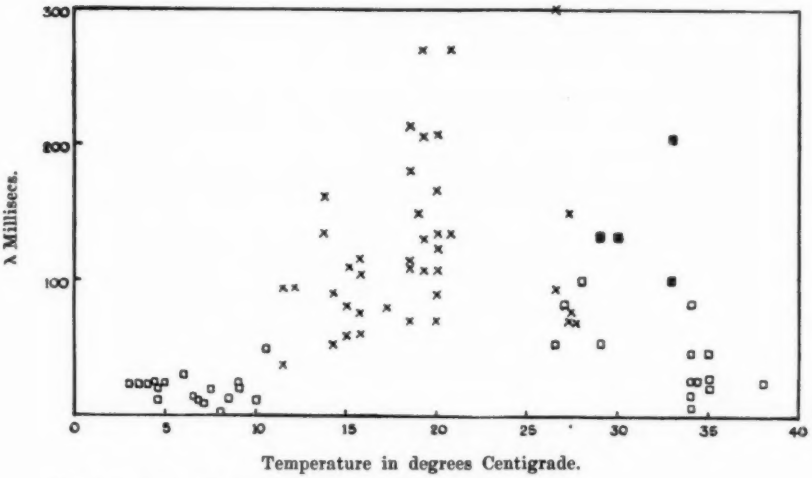

Fig. 2. Shows the values of λ at various temperatures.

× = values of λ obtained at room temperature—i.e., specimens in which there has been no previous temperature alteration.

□ = values to which λ fell when temperature of specimen was altered.

■ = values to which λ rose when temperature of specimen was altered.

The predominant room temperature range was from 15° to 20° C. (Fig. 2).

The initial procedure for each day's work was the same, and the two sciatic-gastrocnemius preparations from the toad were placed in Ringer's solution for 1 to 2 hours before any measurement of λ was made.

The same apparatus was used throughout the series, and, in practically all cases, an accurate time record was kept.

The electrodes used were calomel half-cells, and stimulation of the nerve was carried out by the saline bridge filling the fine plastic tubing which was connected to the calomel half-cell at one end, and whose other end was open. The nerve was supported by the open end of the plastic tube, and could contact, over a narrow area, the saline wick in the lumen of the tube.

Detailed procedure.

The water bath was kept at various test temperatures, and the nerve, after having been tested for λ at room temperature, was placed in the temperature chamber for 5 minutes, at the end of which time the temperature of the nerve, as judged by the thermocouple, was that of the test chamber.

The readings were taken over 5 minutes, after which the preparation was immediately replaced in Ringer's solution at room temperature. The control nerve was then tested in exactly the same way except that the temperature was room temperature.

Half an hour later the test preparation was then measured for λ at room temperature, to determine whether any alteration in λ due to temperature change, was reversible.

It was found necessary to limit the time of readings to approximately 5 minutes, because a progressive change in λ occurs with the nerve in air, irrespective of any temperature alteration.

RESULTS.

At room temperature there is:

(a) Alteration in accommodation with the length of time of soaking. This is usually manifest by λ fluctuating up or down by as much as 25 p.c. during the first 2 to 3 hours after preparation, and then gradually settling over the next four hours to approximately the initial value.

(b) A fluctuation in the initial threshold E_0 —with the length of time of soaking. Over a period of 7 hours E_0 shows a rise by as much as 100 p.c. but settles to somewhere near the initial value at the end of the period. This fluctuation seems to bear no close relationship to the variation in λ with time.

(c) A marked variation from specimen to specimen of the initial values of λ—i.e. before the nerve has been subject to any temperature alteration (see Fig. 2, values marked xx).

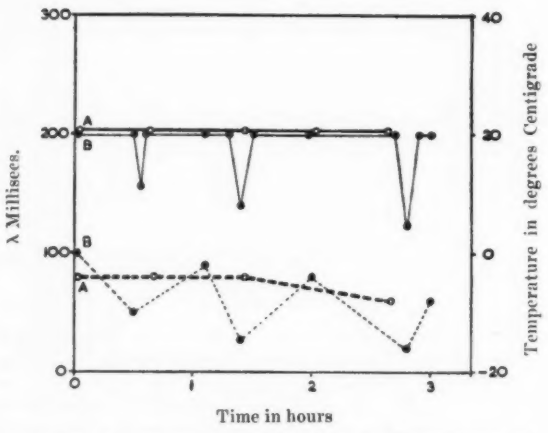

Fig. 3. Shows the alteration in temperature of nerve B, while A from the same toad was kept at an even temperature and also the values of λ for each nerve at the various temperatures, plotted against time in hours after preparation.

o——o = Temperature of A in deg. Centrigrade;

o · · · · o = λ for A in millisecs.

●——● = Temperature of B in deg. Centrigrade;

● · · · · ● = λ for B in millisecs.

Effect of lowering the temperature of toad nerve.

(a) The initial threshold E_0 fell in all cases except one (18 cases).

(b) Accommodation increased (λ decreased) in every case (18 cases). (See Fig. 2). This change occurred irrespective of the time of soaking, and any previous temperature alteration (Fig. 3).

Mean value of λ before reducing the temperature = 119 millisecs.
Mean change in λ .. .. = 93·6 millisecs.
Mean value of reduction in λ/° C. .. .. .. = 11·5 millisecs.
Mean value of temperature before reducing it .. = 16·4° C.

(c) The alteration in λ was reversible—by raising the temperature again, or by replacing the preparation in Ringer's solution at room temperature. In the latter case the nerve was usually retested after half an hour, as shown in Fig. 3.

It will be noted that the reversal in λ is complete, and that the values of λ at room temperature in the test preparation B show the same change with time as in the control preparation A. In other words, the behaviour of the nerve at room temperature in respect of accommodation has not been altered by previous fall in temperature.

Solandt (1936) found that lowering the temperature was accompanied by an increase in λ, but, "a useful reverse could not be obtained."

His changes in temperature were quite slow, whereas in the experiments reported above the change took place over 5 minutes. In a few experiments slow reduction of temperature gave only a fall in λ.

(d) In several experiments the muscle, whilst in Ringer's solution at room temperature, exhibited spontaneous contractions, indicating almost complete absence of accommodation. The nerve muscle preparation was immediately placed in the test chamber which was at a low temperature (4°–6° C.) and, as the temperature of the nerve fell to that of the test chamber, the spontaneous contractions diminished and finally ceased and λ was found to be very low.

Effect of raising the temperature of toad nerve.

Consistency in results was not so evident as in the case of lowering the temperature. A greater number of specimens was therefore tested, in order to assess accurately the effect of raising the temperature.

(a) The initial threshold E_0 increased markedly in all cases except two (28 cases). This alteration was reversible.

(b) The effect on accommodation varied—in 14 cases accommodation increased; in 7 cases it could not be calculated owing to an unusual response of the nerve—see (c); in 4 cases accommodation decreased, and in three cases it remained unaltered. (See Fig. 2.)

In those cases in which accommodation increased with rise in temperature a reversal of the change was obtained in each case in which it was attempted (7 cases).

Fig. 4 shows the typical increase in accommodation (decrease in λ) with rise in temperature and reversibility of the change.

(c) An unusual response was obtained in 7 cases to an increase in temperature (Fig. 5). A curve with its convexity upwards was obtained in plotting the values of E/E_0 against the time constant of current rise, instead of a straight line—(see method as described by Solandt, 1936). In these cases, therefore, there was a sharp decrease in λ (increase in accommodation), fol-

lowed by a gradual increase in λ (decrease in accommodation) over the more slowly building up current rise and, associated with this, could be observed a marked alteration in muscle response.

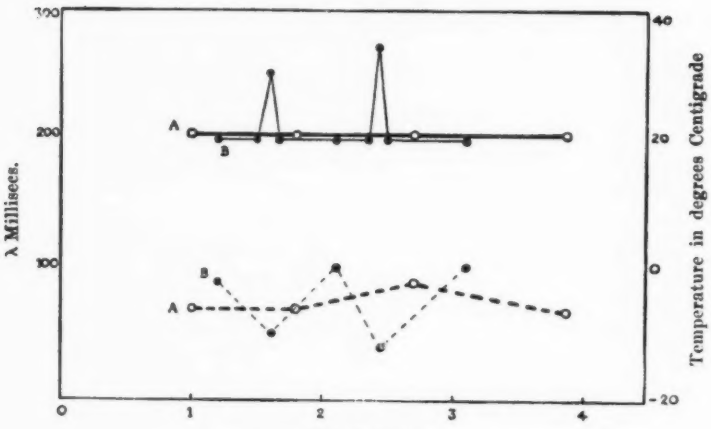

Fig. 4.

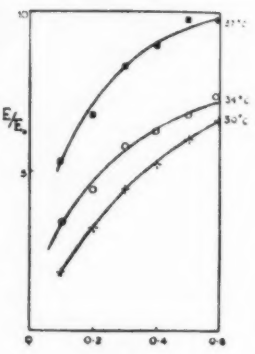

Fig. 5.

Fig. 4. Shows the alteration in temperature of nerve B with corresponding values of λ, whilst nerve A is kept at constant temperature, plotted against time in hours.
●——● = Temperature of B in deg. Centigrade; ● ···· ● = λ for B in millisecs.

Fig. 5. Shows the values of E/E_0 plotted against the time constant of current rise in three different nerves—C, D, E—which showed an unusual response to temperature increase.

C at 30° C., D at 34° C. and E at 37° C.

The latent period was prolonged, as well as the period of contraction and relaxation, and several times repetitive contractions occurred, as is seen with repetitive firing of nerve impulses. This muscle response corresponds exactly with that described by Bernhard, Granit and Skoglund (1942) as occurring in muscle when accommodation processes in nerve have broken down, and stimulation of the nerve under these conditions results in repetitive firing of nerve impulses as demonstrated by action potential tracings.

To summarize then, raising the temperature of toad nerve in these 7 cases produced an increase in accommodation, followed by a sudden breakdown of accommodation with repetitive firing in the nerve.

Distribution and correlation of changes observed.

Temperature-change was accompanied by an alteration in the initial threshold (E_0), the latter decreasing as the temperature was lowered and increasing as the temperature was raised.

On the other hand, there was no correlation between the initial threshold E_0 and the value of λ obtained—so that any variations in λ with temperature cannot be explained by the alteration of the initial threshold, and must therefore be due to an alteration in the accommodation processes of the nerve.

When λ is correlated with temperature (Fig. 2) the distribution of the values of λ has some central tendency with relatively low values of λ at the extremes of temperature and maximum values in the central range of room temperature.

In the central temperature range there is a wide scatter in the values of λ, whereas at either extreme the scatter is very slight.

SUMMARY.

A series of measurements of the time constant of accommodation (λ millisecs.) in toad nerve has been made, at various temperatures ranging from 3° to 38° C.

Lowering the temperature of toad nerve was accompanied by an increase in accommodation (18 cases). These changes cannot be explained by variation in time or alteration in the specimen, because both these factors were fully controlled, and also reversibility was consistently obtained on return of the nerve temperature to normal.

Increasing the temperature of toad nerve was accompanied by an increase in accommodation in 21 out of 28 cases. These changes in accommodation were not all simple, as is shown by an alteration in the gradient of the graph, but in 7 cases were accompanied by an unusual response of the nerve typical of that seen in accommodation breakdown and the graph showed an initial steep gradient followed by a flattening-out over the latter part.

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THE RELATIONSHIP OF AGE TO THE SUSCEPTIBILITY OF MICE TO TWO VIRUSES OF THE COXSACKIE GROUP

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In a preliminary report (Howes, 1951) the isolation of a Coxsackie type of virus from a case of pleurodynia, and the properties of this virus, were briefly described. The virus strain appears to be identical with, or closely similar to, that subsequently isolated by Atkinson, Dineen, and Roberston (1951) from the same case.

Doubt as to the relationship of this virus to the Coxsackie group was felt at first, since, although it was similar in many other respects to some members of this group, weaned mice proved to be susceptible to infection with the virus. However, it has been shown by Pappenheimer, Kunz and Richardson (1951), that in a suitable mouse strain the pathogenicity of the Connecticut 5 strain of "C" virus is no longer restricted to suckling mice, and in addition, it has been shown that for the same virus, predosage of resistant adult mice with cortisone renders these animals fully susceptible to infection (Kilbourne and Horsfall, 1951). It is apparent, therefore, that the virus used in the experiments described in this paper cannot be excluded from the Coxsackie group by virtue of its pathogenicity for adult mice, since the selective pathogenicity for suckling mice is not an altogether suitable criterion for determining whether or not a virus belongs to this group.

This paper is mainly concerned with the variations in the susceptibility of mice to this virus, at different ages, and the effect of cortisone on this relationship.

Relationship of the virus to pleurodynia.

Apart from the case from which the virus was isolated, it has not been possible to demonstrate either rise in neutralizing antibody titre, or the presence of this virus in several cases of pleurodynia subsequently investigated. In addition, all of 14 sera from normal persons from the metropolitan area which were examined by the neutralization technique in weaned mice were found to possess significant levels of neutralizing antibody. These sera were shown to be capable of neutralizing 100 or more LD_{50} of virus. Of 40 sera from normal persons from country areas, 25 were found to neutralize 50 LD_{50}. Although only a small series, these results are suggestive of widespread infection with this virus.

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It is felt that the evidence so far obtained is insufficient to allow definite identification of this virus as an aetiological agent of pleurodynia. Accordingly, to avoid suggesting such a rôle, and following the precedent established by other workers in this field of naming "C" virus strains after the locality from which they were obtained, this virus will be referred to subsequently as the Adelaide 1 strain.

Materials and Methods.

Viruses. For Adelaide 1, 9th suckling mouse passage virus was used in Experiment 1, while subsequent experiments were carried out using the one batch of 10th suckling mouse passage virus. Connecticut 5 was used as 9th suckling mouse passage virus.

Virus suspensions: Preparation of virus suspensions was carried out in a manner similar to that recommended by Melnick and Ledinko (1950). Mice less than hours old were inoculated intraperitoneally with 0·05 ml. of a 10 p.c. muscle-bone suspension of infected mice. Two to three days later, when sick, the mice were killed, washed in ether, and head, skin, and viscera removed. The carcasses were then washed in ether, and 20 p.c. suspensions in 10 p.c. rabbit serum saline prepared by grinding with silica. Following overnight extraction at 4° C., the suspensions were centrifuged at 5–6,000 r.p.m. in a refrigerated angle-head centrifuge for 2 hours. The supernatant was stored in 1 ml. amounts in a CO_2 cabinet until use.

Dilutions. All virus dilutions were made in 10 p.c. rabbit serum saline to which were added penicillin and streptomycin at a concentration of 500 units per ml.

Cortisone. Cortisone acetate (Merck) was used. Dilutions of cortisone were prepared in saline.

Mice. All mice used in these experiments were from an inbred strain of white mice maintained at this Institute. Ages of suckling mice were known to within ± 12 hours and of weaned mice, to within ± 24 hours. To avoid as far as possible variations in susceptibility of different litters of the same age, all suckling mice of each age group were pooled, and after mixing, redistributed at random in approximately equal numbers to the does from which they came. Litters 48 hours or more old, of only two or three in number, and as a result, usually above normal weight were discarded. Where a litter was undernourished, both doe and litter were discarded.

In an experiment carried out prior to this series, no demonstrable effect of weaning on the virus titres obtained was found. Parallel titrations of Adelaide 1 in groups of weaned and unweaned 20–22-day-old mice, using 5 or more mice per dilution, gave an LD_{50} titre of $10^{-6 \cdot 5}$ in both cases. Accordingly, for convenience, surviving suckling mice in each group were weaned as they reached an age of three weeks.

Cortisone and saline inoculations. Before administering cortisone, the mean body weight of mice in each group was determined, and the dosage calculated so that in each experiment a constant amount of cortisone per gram body weight was used. In each case, the required amount of cortisone was contained in 0·05 ml. and was injected intraperitoneally. In the experimental series using Adelaide 1, in which no cortisone was administered, an equivalent volume of saline was given intraperitoneally.

Detection of fat replacement of pancreatic tissue by bulk staining. A method similar to that used by Pappenheimer, Kunz, and Richardson (1951) was used. At the end of the period of observation, surviving mice were killed with ether, and the pancreas of each was dissected out and fixed overnight in Bouin's fluid. The organs were then stained in bulk for 1 to 2 hours in a saturated solution of Scharlach R. in equal parts of 70 p.c. ethyl alcohol and acetone.

At the end of this period, after briefly washing in 70 p.c. alcohol, each pancreas was examined for the presence of the scarlet areas denoting the presence of fat.

Microscopic examination of pancreases for the presence of fat. The pancreases of a few groups of surviving mice were examined after paraffin sections stained by haematoxylin and eosin had been made in place of bulk staining.

Period of observation. Suckling and weaned mice were examined daily for periods of 25–32 days and 35–40 days respectively, with the exception of Experiment I. These times were chosen to allow deaths from all but the more chronic infections to take place, and to allow a period of sufficient length for the replacement of necrotic pancreatic acinar tissue by fat to reach completion. A longer period was allowed for weaned mice since the incubation period was prolonged beyond that seen in suckling mice.

That only 3 of 464 deaths in suckling mice occurred later than 20 days after inoculation, and none of the 120 deaths in weaned mice occurred later than 30 days, was considered to justify the lengths of the periods selected. Only two were obviously sick at the time of sacrifice, and these had shown the hunched attitude and ruffled fur typical of the desease in weaned mice for 21 days without change. Further, in those groups of mice in which microscopic examination of the pancreas was made, no evidence of stages in the evolution of pancreatic lesions earlier than that of fat replacement was seen.

Technique of virus titrations. Serial tenfold dilutions of a 10 p.c. suspension were used in each experiment, the one series of virus dilutions being used for all age groups.

For each dilution, a dose of 0·05 ml. given intraperitoneally was used for all age groups.

Approximately equal numbers of mice were allocated to each dilution, except that whenever possible at critical dilutions, two litters were used instead of one.

For suckling mice, the number of mice inoculated for each dilution varied between 5 and 8 for different age groups, usually 5 or 6, while for weaned mice, 5 mice per dilution were used for all age groups.

The numbers of dilutions tested in different age groups varied from 3 to 6, usually 4 or 5, as dictated by the supply of litters.

Calculation of virus titres. The 50 p.c. lethal dose (LD_{50}) of virus, and the 50 p.c. infective dose (ID_{50}) were calculated wherever possible by the method of Reed and Muench (1938). LD_{50} titres were calculated from the numbers of deaths which occurred during the period of observation, while ID_{50} titres were calculated by adding to the number of deaths at each dilution the number of mice which were found by bulk staining or microscopic examination to have suffered damage to pancreatic acinar tissue.

Neutralization tests. Undiluted serum inactivated at 56° C. for 30 minutes was mixed with equal volumes of a series of virus dilutions and placed in a water bath at 37° C. for 2 hours. Both suckling and weaned mice received an 0·05 ml. dose of the virus serum mixture intraperitoneally. Suckling mice were examined daily for 14 days, and weaned mice for 21 days. Normal mouse serum was used as control in the three tests. Neutralization indices were calculated from LD_{50} titres.

In both the age-susceptibility titrations and neutralization tests deaths occurring during the first 48 hours after inoculation were considered to be non-specific.

EXPERIMENTAL.

The relationship between age and susceptibility to lethal and sublethal infection with Adelaide 1.—Experiments I and II.

In these experiments, parallel titrations in different age groups of mice from newly-born to adult, were carried out on suspensions of 9th and 10th suckling mouse passage virus respectively.

TABLE 1.

Lethal and sublethal infection of suckling and weaned mice by Adelaide 1 and Connecticut 5.

	Age when Inoculated	Adelaide 1. Experiment I Virus only	Experiment II Virus+Saline			Experiments III and IV* Virus+Cortisone (0·05 mg./gm.)			Cortisone Controls†		Connecticut 5 Virus only		
	(days)	LD_{50}‡	LD_{50}	ID_{50}	ID_{50}–LD_{50}	LD_{50}	ID_{50}	ID_{50}–LD_{50}	Experiments III and IV		LD_{50}	ID_{50}	ID_{50}–LD_{50}
Suckling mice.	0–1	6·83	6·83	>7·00	>0·17	6·83	6·83	0·00	2/12	0/10	7·67	7·67	0·00
	2–3		6·37	6·57	0·20	7·24	7·24	0·00	3/8	0/5			
	3–4	5·31	5·27	5·81	0·54						6·52	6·62	0·10
	4–5					7·80	8·50	0·70	3/18	0/15			
	5–6	3·35	4·13	4·75	0·62	6·37	>7·00	0·43	1/16	0/15			
	6–7	(approx.)	3·71	4·00	0·29								
	7–8	2·00									2·00	5·55	3·55
	8–9	(approx.)				6·29	7·50	1·21	0/6	0/6	(approx.)		
	9–10		1·24	4·78	3·54	{6·23	{7·34	{1·11	{2/19	{0/17			
			(approx.)			{4·88	{6·29	{1·41	{0/5	{0/5			
	10–11		<1·00	>6·00	>5·00						2·00 (approx.)	5·85	3·85
	11–12	<3·00				6·50	6·69	0·19	0/5	0/5			
	12–13		>5·00	>5·00	—								
	14–15	5·25									5·83	6·38	0·55
	15–16		5·29	5·47	0·18	<6·00	6·50	>0·50	0/5	0/5			
	16–17										5·30	5·85	0·55
	17–18	6·33	6·00	6·50	0·50	6·31	6·31	0·00	0/5	0/5			
Weaned	21–23	5·46	5·78	6·25	0·47	5·50	6·69	1·19	0/5	0/5	4·50	5·36	0·86
	28–29	6·37											
	34–39	4·25	4·50	4·62	0·12	5·00	5·00	0·00	0/5	0/5			
	41–42	4·62											
	61–71		4·42	4·83	0·41	3·83	4·17	0·34	0/5	0/5			
	81–82	4·60											
	102–103		4·83	4·83	0·00								

* LD_{50} and ID_{50} titres in 9–10-day mice for Experiment IV are given below those for Experiment III. LD_{50} and ID_{50} titres for untreated 21–23-day-old mice used as virus controls in Experiment IV were 5·68 and 5·75 logs respectively.

† Fractions on the left of columns indicate the proportion $\frac{\text{number dead}}{\text{number inoculated}}$, those on the right of columns. $\frac{\text{number of survivors with pancreatic lesions}}{\text{number of survivors}}$.

‡ Titres are expressed as the negative logarithms.

In Experiment I, 7 groups of suckling mice and 5 groups of weaned mice were used in titrations on 9th passage virus. Mice were observed for 27 days after inoculation and then discarded.

Experiment II was carried out in conjunction with Experiment III and all mice were injected with 0·05 ml. saline intraperitoneally prior to virus inoculation. Ten groups of suckling mice and five groups of weaned mice were used. Suckling mice were observed for 25-32 days and weaned mice for 39 days. Survivors were killed, and their pancreases removed. All survivors of the 0–1 day age group, and the survivors of the 9–10 day old group inoculated with a 10^{-4} dilution of virus were examined microscopically. The pancreases of the remaining mice were examined by bulk staining.

The LD_{50} and ID_{50} titres for these experiments are given in Table 1.

The LD_{50} titres obtained showed a progressive fall in value as age was increased from 0–1 to 10–11 days. In both experiments virus titred 6·83 logs in mice in the first day of life, but the titre thereafter fell rapidly. In the restricted series of age groups used in Experiment I, a minimum titre of approximately 2 logs was obtained in 7–8 days old mice, but the results of Experiment II indicated that mice 10–11 days old were even more resistant, as although virus dilutions from 10^{-1} upwards were tested insufficient deaths occurred for an end point to be obtained.

Following this phase of resistance, the incidence of lethal infection was found to rise very rapidly, so that in mice 12-13 days old, the end point lay above the highest dilution of virus tested, 10^{-5}.

High virus titres were again obtained as the age of mice increased from this point. In Experiment I, the highest values were obtained in 17–18 and 28–29 day old mice, these titres being 6·33 and 6·37 logs respectively, approximately 0·4 logs less than the titre of virus in newborn mice. In Experiment II, a peak value of 6·00 logs was obtained in mice 17–18 days old.

Following the high titres obtained in mice 17–29 days old, titres of from 4·4 to 4·9 logs were obtained in mice from 5–15 weeks old. Although the fall in titre may be due in part to the increasing weight of these animals while a constant inoculum was used, the overall fall in titre appears to be too great to be considered due to this factor alone, and is taken as indicating a slight fall in susceptibility below that for the former group.

Although in both experiments the variation in titre with increase in age followed a fairly steady course with no marked deviation from the general trend, the values obtained for the LD_{50} virus titre as age increased from 3 to 11 days were progressively less reliable and could only be considered as approximations since the fall in the proportion of deaths in the various test groups did not follow a smooth course as virus concentration was decreased. This

TABLE 2.

*Death and infection in suckling mice in relation to virus concentration.**

Virus Dilution	Age at time of inoculation (days)											
	Adelaide 1						Connecticut 5					
	2 – 3		6 – 7		10 – 11		15 – 16		3 – 4		7 – 8		10 – 11		14 – 15	
10^{-9}											0/4	0/4			0/6	0/6
10^{-8}											0/6	0/6	0/5	0/5	0/6	0/6
10^{-7}	0/3	1/3					1/6	1/6	1/5	1/5	1/4	1/4	1/5	2/5	1/5	1/5
10^{-6}	4/5	4/5	2/7	3/7	2/5	3/5	1/6	1/6	5/6	6/6	1/5	2/5	0/5	2/5	3/6	4/6
10^{-5}	4/4	4/4	1/7	2/7	0/6	5/6	3/6	4/6	5/5	5/5	1/6	4/6	1/5	5/5	3/5	5/5
10^{-4}	5/5	5/5	1/6	1/6	0/6	6/6	6/6	6/6	6/6	6/6	0/6	5/6	1/4	4/4	6/6	6/6
10^{-3}	3/3	3/3	5/7	6/7	0/6	6/6	6/6	6/6	5/5	5/5	3/6	6/6	1/5	3/5	5/5	5/5
10^{-2}			8/8	8/8	0/5	5/5					1/4	4/4	2/5	5/5		
10^{-1}			5/5	5/5	1/5	5/5					1/5	1/5	2/5	5/5		
LD_{50}	6·37		3·71		<1·00		5·29		6·52		2·00 (approx.)		2·00 (approx.)		5·83	
ID_{50}	6·57		4·00		>6·00		5·47		6·62		5·55		6·22		6·38	

* The fractions on the left of columns indicate the proportion $\frac{\text{number dead}}{\text{number inoculated}}$; the fractions on the right indicate $\frac{\text{number infected}}{\text{number inoculated}}$.

was most apparent in mice 7–11 days old, in which almost no relation between the two could be detected in either experiment (Table 2).

Examination of the pancreases of survivors in Experiment II showed that the incidence of deaths in the various age groups did not indicate the true level of susceptibility to virus infection.

In all groups except the 102 to 103 day old mice, survivors were found to show evidence in at least 1 case in each age group of fat replacement of acinar tissue. This was most frequent in mice which had survived infection with concentrated virus suspensions in the 9–10 and 10–11 day age groups.

Occasional survivors of infection with the lower virus dilutions in 9–10 day old mice gave no evidence of infection by the method of bulk staining, suggesting that some mice of this age may be completely resistant to infection.

Apart from the 9–10 and 10–11 day age groups and in contrast to LD_{50} end points, ID_{50} titres were relatively sharp. The lowest value for the ID_{50} titre of virus was 4·00 logs, obtained in 6–7 day old mice, this titre being over 3 logs less than in mice in their first day of life and over 2 logs less than in mice in their 11th day of life. The relationship of age to susceptibility for mice greater than 17 days old was not materially different from that indicated by LD_{50} values, ID_{50} titres being greater than LD_{50} titres by from 0 to 0·5 logs.

In mice surviving infection, the amount of acinar tissue destroyed varied from approximately 5 p.c. to 90 p.c. or more, all survivors being found to possess some undamaged tissue, but as in the case of the proportion of deaths occurring, no evidence was obtained of any correlation between the amount of virus inoculated and the amount of acinar tissue destroyed.

In all, the results indicated that in this strain of mice there is a slight fall in susceptibility to infection during the first seven days of life, this being reflected by lowered ID_{50} virus titres in mice during that period. Susceptibility to infection is at its lowest on the 7th day, but by the 11th has again risen to a value approaching that for newborn mice. Apart from this phase of reduced susceptibility, the incidence of infection showed a relatively gradual fall with increase in age. The LD_{50} titres obtained in mice 15 weeks old were reduced to a value only 2 logs below that for mice 0–1 days old.

However, for a period of approximately 4 days early in the second week of life, although small amounts of virus are capable of initiating infection, in only a relatively small proportion of mice (at all virus concentrations) is the involvement of pancreatic tissue sufficiently extensive for the reduction in secretory activity to be sufficient to lead to death of the animal. Consequently, although infection is almost as frequent as in mice of ages on either side of this period, the number of deaths which occur is markedly less.

The effect of cortisone on the age-susceptibility relationship for Adelaide 1.—Experiments III and IV.

Experiment III was carried out at the same time as Experiment II, using the same series of virus dilutions. Two to four hours prior to inoculation with virus, mice received 0·05 mg. cortisone per gm. body weight intraperitoneally. For each age group a group of at least five mice was used as a cortisone control, these mice receiving the same amount of cortisone as virus inoculated animals. A total of nine age groups of suckling mice were used, a corresponding age group being found in the virus-saline series of Experiment II, for six of these groups. Mice were observed for the same length of time as in Experiment II.

In contrast to untreated mice, no comparable fall in the incidence of lethal infection was apparent in 8–12 day old mice. From an initial titre of 6·83 logs in newborn mice, the titre increased to between 7·2 and 7·8 logs in the next two groups of mice; from the latter value the titre fell approximately 1·4 logs to a value of 6·37 logs in 5–6 day old mice. However, the high titres obtained in 2–5 day old mice are in doubt, as a small proportion of deaths occurred in the cortisone treated controls. LD_{50} titres in suckling mice older than 5–6 days showed little variation from the titre obtained in 5–6 day old mice.

As in untreated mice between 8–12 days old, the titre in 9–10 day old treated mice was unsatisfactory since there were less than 100 p.c. deaths over a wide range of dilutions, although the approximate titre was over 5 logs greater than in untreated mice of the same age. In mice outside the 8–12 day age range, however, end points obtained in treated mice were sharper than those obtained in untreated mice.

ID_{50} end points were sharp in all age groups. In three of these groups, LD_{50} and ID_{50} titres were identical, and in others, differences between the two end points of up to 1·4 logs were seen. The greatest difference was seen in 8–10 day old mice, which suggested that the dosage level of cortisone used was insufficient to raise the proportion of deaths to infections to the level seen in untreated mice outside the 8–12 day age range.

In Experiment IV, virus was titrated in weaned mice pretreated (at the same dosage level as in Experiment II) with cortisone. Virus was also titrated in 9–10 day old treated mice and untreated newly weaned mice for purposes of comparison with Experiment III.

Although the LD_{50} and ID_{50} titres in 9-10 day old treated mice were lower than in the previous experiment by 1·35 logs and 1·05 logs respectively the titres in untreated 21–23 day old mice indicated that the virus concentration of the suspension used was not appreciably different from that used in the previous experiment.

As for untreated weaned mice, the virus titre fell gradually as the age of mice was increased, the LD_{50} virus titre of 5·50 logs for 21–23 day old mice being 1·67 logs higher than for 70 day old mice, while the ID_{50} titre of 6·69

logs was 2·52 logs higher. Taken as a whole, neither the LD_{50} nor the ID_{50} titres for treated weaned mice showed any appreciable variation from the trends seen in untreated weaned mice (Table 1).

The results of the two experiments on pretreatment with cortisone indicated that in suckling mice the progressive lowering of susceptibility to infection in the first 7 days of life, and the markedly lowered incidence of *lethal* infection in mice during the next four days of life was to a large extent counteracted by predosage of mice with cortisone.

In contrast to this neither susceptibility to infection, nor the relative incidence of lethal infection appear to be altered appreciably in weaned mice by the administration of cortisone.

The development by Adelaide 1 of pathogenicity for weaned mice.

Since Coxsackie viruses have, until recently, been considered in the laboratory to be essentially pathogens of *suckling* mice and hamsters, it was thought that the pathogenicity of the Adelaide 1 strain for weaned mice may have been acquired since isolation, during suckling mouse passage.

This possibility was tested by comparing ID_{50} titres of 3rd, 9th and 10th suckling mouse passage virus in both weaned and suckling mice.

Two titrations of 3rd passage virus in weaned mice gave titres of 4·50 and 4·41 logs, compared with a titre of 6·48 in 0–1 day old mice. Ninth passage virus titred 5·46 and 6·83 logs in weaned and suckling mice respectively while tenth passage virus titred 5·78 and 6·83 logs in the two groups respectively.

Comparison of these figures showed that the difference between titres in suckling and weaned mice decreased from 2·03 logs for 3rd passage virus through 1·37 logs for 9th passage virus to 1·05 logs for 10th passage virus. These results suggest that in suckling mouse passage, adaptation to the suckling mouse takes place more rapidly than adaptation to the weaned mouse.

The age-susceptibility relationship for Connecticut 5.

Pathogenicity of the Connecticut 5 strain for weaned mice of the strain maintained at this Institute was first noted in attempts to prepare antiserum against the virus, in adult males. The similarity of the disease in adult mice, and the marked tropism for the pancreas suggested a relationship of the Adelaide 1 strain to Connecticut 5, in these properties at least. Pappenheimer, Kunz and Richardson (1951) have shown that with their strain of mice seven day old mice are more resistant to lethal infection than either newborn or fourteen day old mice.

To investigate the relationship between age and susceptibility to Connecticut 5 in our strain of mice, 10th suckling mouse passage virus was titrated in six age groups of suckling mice, and one age group of newly weaned mice. A similar age-susceptibility relationship to that found for Adelaide 1 was apparent.

LD_{50} titres (Table 1) in 7–8 and 10–11 day old mice were 5·67 logs lower than the titre in newlyborn mice. After a second peak value of 5·83 logs in 14–15 day old mice, the virus titre fell to 4·50 logs in newly weaned mice 20–21 days old.

TABLE 3.

Serological comparison of Adelaide 1 and Connecticut 5.

Neutralization test in	Suckling mice		Weaned mice	
Antiserum	Adelaide 1	Connecticut 5	Adelaide 1	Connecticut 5
Virus				
Adelaide 1	100,000*	0	6,600	5
Connecticut 5	13	42,000	N.D.†	N.D.

* Neutralization Index. † Not done.

Virus end points in 7–8 days and 10–11 days old mice were only rough approximations since, as for Adelaide 1, the proportion of deaths at each virus dilution bore little relationship to the virus concentration (Table 2). The relationship between death and infection in various age groups is indicated in Fig. 1. Again, as for Adelaide 1, no correlation could be detected between the amount of acinar tissue destroyed and the amount of virus in the inoculum.

ID_{50} titres were calculated after examination of the pancreases of survivors, and it was found that this titre was at a minimum in 7–8 day old mice, being 5·55 logs in this case, compared with 7·67 and 6·38 logs in 0–1 and 14–15 day old mice.

In the two groups of mice 7–8 and 10–11 days old, although only a small proportion of mice inoculated with the lower dilutions of virus died, a high proportion of survivors was found to have suffered an inapparent infection, resulting in partial destruction of the pancreas.

This relationship contrasted with that found in other age groups (Fig. 1).

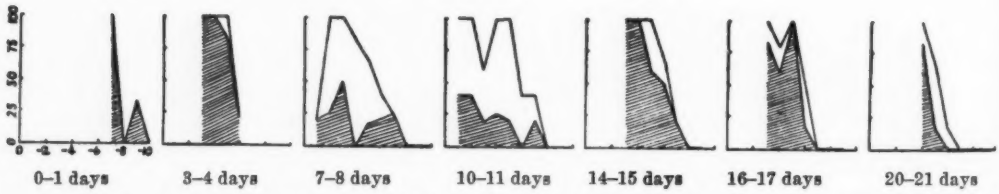


Fig. 1. Variations in percentage of deaths and total infections in mice of different ages in relation to virus dilution, Connecticut 5.

Ordinate: Percentage of deaths or total infections. Abscissae: Log virus dilution.
Percentage deaths is represented by lower line enclosing shaded area.

Taken as a whole, the results indicated that susceptibility to infection is at a minimum in 7–8 day old mice, although the period of partial susceptibility reflected by a greatly lowered incidence of deaths is maintained until the 11th day of life at least.

Serological relationship of Adelaide 1 to Connecticut 5.

Although they are similar in many properties, no serological relationship between the two viruses has yet been found. In cross neutralization tests in suckling mice using sera obtained from three month old mice which were still alive three weeks after receiving concentrated virus suspensions, high antibody titres were obtained with antisera to the homologous virus, but not with antisera against the heterologous strain.

Similar results were obtained when further antisera from the same mice were tested by the neutralization test in weaned mice, using the Adelaide 1 strain of virus (Table 3).

DISCUSSION.

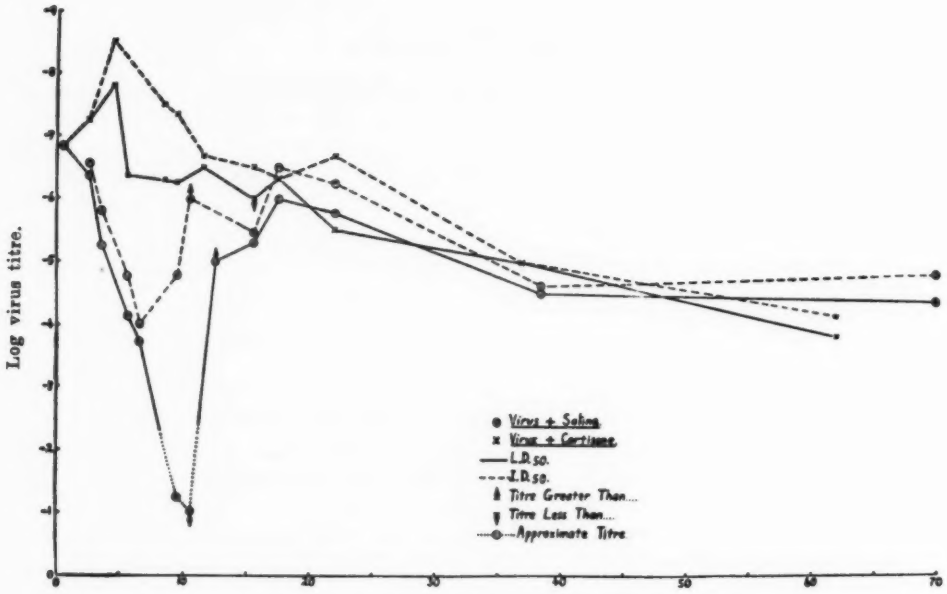
Preliminary investigations on the susceptibility of mice to the Adelaide 1 strain using a limited number of age groups had made apparent the susceptibility of newly weaned and adult mice to this virus, and gave the impression of a gradual fall in susceptibility with increase in age.

However, in quantitative experiments in which the virus titre obtained in mice of various ages has been used as an index of susceptibility, it has been shown that during the first two weeks of life rapid changes in the incidence of both lethal and sub-lethal infection occur as age increases (Fig. 2).

Taking death or the presence of areas of fat replacement in the pancreas as criteria of infection, it is apparent that susceptibility to infection declines moderately rapidly from its highest value in newborn mice, reaching a minimum in mice in their eighth day. Following this phase of declining susceptibility, susceptibility to infection returns rapidly at first, and then more gradually, to a new peak value in mice in the third or fourth week of life. With further increase in age, a continual gradual decline in susceptibility is again apparent.

Comparison of the incidence of deaths and infections indicates that although the incidence of lethal infection is only slightly less than that of total infections (lethal and sub-lethal) and falls at approximately the same rate during the first days of life, the former does not reach its minimum value until the eleventh day. Thus during a period from the eighth to the eleventh day, although susceptibility to infection is increasing, the proportion of infected animals which die is falling, suggesting that, although the virus has

decreasing difficulty in initiating infection, the subsequent development of the pancreatic lesion is increasingly adversely affected by developmental changes in the host.



Age at time of inoculation (days).

Fig. 2. Relationship of age to the occurrence of lethal and sub-lethal infection with Adelaide 1, and the effect of Cortisone.

Emergence from this phase is rapid, and by the 16th day the difference between LD_{50} and ID_{50} titres is again of the same order as that found in mice during the first phase. With further increase in age only minor changes in the relationship of these titres occur.

The effect of cortisone on the age-susceptibility relationship for Adelaide 1 in this strain of mice is most marked during the second phase of rising incidence of infection but falling incidence of deaths, this phase being almost inapparent in treated mice (Fig. 2).

The conclusion drawn from these results is that the action of cortisone on the development of the disease in mice is largely concerned with those stages in the development of the pancreatic lesion subsequent to the actual infection of the pancreatic cells. Whether the elimination by cortisone of the phase of reduced death incidence is indicative of reduced functional activity of

the adrenal cortex during this period of development, or is indicative merely of a reduction in the ability of pancreatic tissue to support rapid virus propagation after infection has taken place, a state reversed by a level of cortical hormones in excess of that normally present was not indicated in these experiments. However, the results indicate the desirability of investigating possible variations in adrenal cortical activity, the rate of virus propagation in both muscle and pancreas, and the relative importance of muscle and pancreatic lesions as the cause of death in suckling mice in the first week of life.

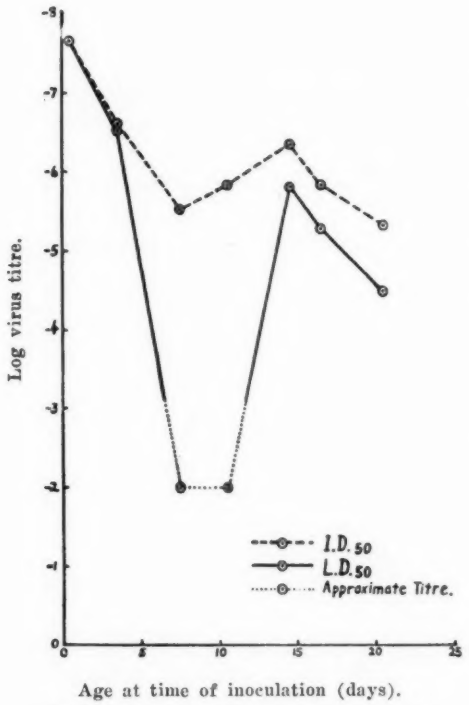


Fig. 3. Age susceptibility relationship for Connecticut 5.

Investigation of the age-susceptibility relationship for the Connecticut 5 strain indicated that the relationship is essentially the same as that for Adelaide 1 (Fig. 3) and is similar to that found for Connecticut 5 by Pappenheimer, Kunz and Richardson (1951). Although no serological evidence of a relationship between the two viruses has been obtained, this similarity suggests that these viruses may otherwise be closely related.

Until recently the selective pathogenicity of Coxsackie viruses for *immature* mice and hamsters (Gifford and Dalldorf, 1951), has been stressed, but the demonstration of the susceptibility of mature mice to strains of this virus group in Pappenheimer's and this laboratory indicates that this selective pathogenicity is not suitable as a criterion for inclusion of a virus strain in the Coxsackie group. Whether or not a virus strain satisfies this criterion is probably to a large extent dependent on the characteristics of the strain of mice used.

Although adult as well as suckling mice are susceptible to the Adelaide 1 strain, the virus is selectively pathogenic for *suckling* rats. Beginning with 10th suckling mouse passage virus, the Adelaide 1 strain has been transmitted

through four serial passages in 0–1 day old suckling rats of the inbred strain of hooded rats bred in this Institute, with the production of death and occasional paralysis. The ID_{50} titre of muscle from infected suckling rats of the third passage was 6·68 logs in newly weaned mice, which was slightly higher than that usually found for suckling mouse muscle. Weaned rats of this strain showed no signs of disease, but a low titre viraemia lasting several days has been detected.

SUMMARY.

Results of an investigation into the age-susceptibility relationship in mice to infection with the Adelaide 1 and Connecticut 5 strains of Coxsackie viruses are described.

For the Adelaide 1 strain 5 stages in this relationship during growth are discernible:—

From 0–8 days, falling susceptibility to infection with no reduction in the proportion of deaths to infections.

From 8–11 days, rising susceptibility to infection with a marked fall in the proportion of deaths to infections.

From 11 to 13 days, continued rise in susceptibility to infection with a very rapid rise in the proportion of deaths to infections.

From 13 days onwards, a rise in both factors to a peak during the third or fourth week.

From this peak, a gradual fall with further increase in age.

A similar relationship is evident for Connecticut 5.

In mice pretreated with cortisone and infected with Adelaide 1, stages 2 and 3 are no longer apparent.

The above findings are discussed, and the pathogenicity of the Adelaide 1 strain for suckling rats is briefly recorded.

Acknowledgments. I am indebted to Dr. J. A. R. Miles for his valuable advice and criticism, and to Mr. K. R. Grosser for technical assistance. I am further indebted to Dr. J. L. Melnick who very kindly supplied the Connecticut 5 strain used in these experiments.

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STUDIES ON NORMAL AND PATHOLOGICAL SERA

I. MEIOSTAGMIN REACTIONS

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The ultimate aim of this work is to devise practical serological tests for some forms of malignant tumours in man. The many failures of tests for this purpose (Stern and Willheim, 1943) need not be discouraging as they merely indicate that further attempts should be made to correlate new compounds, chemical systems and physical phenomena with the statics (existence) and dynamics (biochemical processes) of cancer. To simplify the language of this and future communications, we shall use the term "cancer" to include all forms of malignant proliferation in humans without histological, anatomical or aetiological distinctions. It is expected, however, that these distinctions will have to receive serious consideration before recommending results for practical use. With the accumulation of statistical data we hope to reach a stage where it is profitable to replace the general term "cancer" by more specific terms. In the meantime we assume as a working hypothesis that there may exist common chemical and/or physical features in all forms of cancer.

As a further working hypothesis, it is assumed that cancer is not likely to be identified with any simple phenomenon of physiological chemistry, e.g. excess or deficiency of some pathological or normal compound, but that the decisive changes affect a chemical system of great but as yet undefined complexity. This points to the necessity of using research methods capable of dealing with chemical systems rather than single compounds. The methods of surface and colloid chemistry and those of immunology appear to be the best from this point of view.

It is also postulated that blood as a system must be affected by contact with malignant sites. Although the great chemical and physical buffering powers of blood may prevent detectable or significant changes of a static nature, previous demands made on the buffering powers must reduce the latter against new demands of a totally different kind. In general one cannot predict whether an alteration of buffering powers will be divided evenly over the whole plasma or concentrated in certain blood fractions. The latter possibility is more likely but it requires a number of separate research projects. For reasons of convenience, serum is being considered in the first project.

In view of an excellent survey of the general principles of cancer diagnostic tests (Dunn and Greenhouse, 1950), it will be sufficient to restrict our comments to the view that such tests need not be perfect to be of practical value.

Meiostagmin reactions with acetamide.

Meiostagmin reactions (MR) are diagnostic tests based on observations of decreased surface tension (increased surface activity). In a restricted sense the term MR refers to the cancer tests originated by Ascoli and Izar which were extended and modified by many later workers (Stern and Willheim, 1943). All the older MR amount to a determination of the buffering power of serum in the presence of powerful surface active agents.

Polya and Dunn studied MR in the presence of substances with surface activities inferior to serum. When serum is diluted with a 5 p.c. aqueous solution of acetamide the surface tension versus dilution curve passes successively through a maximum and a minimum, and rises slowly from the latter towards the surface tension value of 5 p.c. acetamide solution. The difference between the surface tension values in dyne/cm. units of undiluted serum and serum diluted to the point where the minimum value of the curve is observed was termed G-value. This was found to be negative in normal and positive in cancer sera. A later experimental survey confirmed the published data and presented the following statistical characteristics.

In a group of 84 cancer and 43 normal sera, the cancer sera had a mean G-value of $+1{\cdot}3$ against $-0{\cdot}6$ for normal sera. The standard deviations ($1{\cdot}7$ and $1{\cdot}9$ respectively) are rather large but the difference of means with standard error is $1{\cdot}9 \pm 0{\cdot}34$ giving d/se of $5{\cdot}6$, and therefore the difference of the means is highly significant. Both the χ^2 and variance tests lead to probability points of well below $0{\cdot}1$ from which one may regard the difference between the G-values for cancer and normal sera as highly significant.

Tabulations of clinical data and notes on interfering conditions (pregnancy, early treatment with insulin, treatment with morphine or digitalis, thyreotoxicosis and certain anemias) are available (Polya and Dunn, 1950; Parkes, 1950) but have little bearing on the subject of the present communication. The technique is being adapted for the purpose of mass surveys at present and a detailed discussion of the results of MR on normal, cancer and other pathological sera must be deferred until more statistical material is available.

Modifications of acetamide in the MR.

Unless great care is taken in purifying acetamide for the MR, results cannot be duplicated and the curves display multiple maxima (as in the case of leukemias). Recrystallization of distilled acetamide from ethyl acetate and benzene followed by the complete removal of traces of solvents in vacuum over

paraffin chips affords a good reagent of good keeping quality. If distilled acetamide is purified by recrystallization from acetone or precipitation from acetone solution with ether or petroleum ether followed by the complete removal of solvents a product (acetamide-b) is obtained which is identical with acetamide obtained by the first method (acetamide-a) as regards melting point and analysis but is unsuitable for the purposes of the MR. Acetamide-b in 5 p.c. aqueous solution is more surface active than acetamide-a. The results cannot be duplicated accurately but the surface tension values found for various preparations of acetamide-b are on the average 5 p.c. lower than those of corresponding solutions of acetamide-a which has a surface tension of 70·1 dyne/cm in 5 p.c. aqueous solution. In one instance a sample of acetamide-b has changed spontaneously into acetamide-a after standing for a few weeks.

One may consider the tautomeric forms $CH_3C(:O)NH_2$ (I), $CH_3C(:NH)OH$ (II) and the often ignored possible tautomer $CH_2:C(OH)NH_2$ (III) for acetamide. The overwhelming bulk of acetamide is best represented by I (Richards and Thompson, 1947; Richards, 1948) and slight variations of the tautomeric equilibrium insufficient to alter the m.p. would not affect the MR if its effects were due solely or mainly to I. It is impossible to decide at present whether II or III or some particular equilibrium between them is required for the MR. The following considerations, however, may guide further work in this field. Tautomeric forms related to both II and III exist in diacylimines, e.g. diacetimide in the form $CH_2:C(OH).N:C(OH)CH_3$ (Polya and Spotswood, 1949 and 1951). Diacetimide in 5 p.c. aqueous solution has a lower surface tension than acetamide (67·8 dyne/cm.) and gives erratic results in the MR, somewhat like acetamide-b. Some of the difficulties in using diacetimide in the MR appear to be due to the presence of iso-diacetimide, $CH_3C(:NH)OAc$ (Dunn and Polya, 1950; Padgham and Polya, 1951), which is more closely related to II than to III. This might indicate that treatment with acetone increases the concentration of II at the expense of III. The carbonylic character of amides, as assessed by the success of modified Perkin reactions is presumably due to forms related to III (Polya and Spotswood, 1951; Parkes, Polya and Spotswood, 1952) and hence the preferential removal of III by acetone is plausible. As reported before, erythrocytes interfere with the MR but their effect may be counteracted by small amounts of sodium cyanide (Polya and Dunn, 1950). If this effect is ascribed tentatively to carbonic anhydrase, one would expect a greater action on III than on II on structural grounds.

Surface tensions of amides in aqueous solution.

In order to facilitate the investigation of amides other than acetamide in the MR the variation of the surface tension of some soluble aliphatic amides

with concentration has been studied. The results were expected to follow the Freundlich law

$$\frac{\gamma_o - \gamma_c}{\gamma_c} = SC^{1/n} \quad . \quad . \quad . \quad . \quad . \quad 1$$

and the Szyszkowski law

$$\frac{\gamma_o - \gamma_c}{\gamma_c} = B \log_{10}(C/A + 1) \quad . \quad . \quad . \quad . \quad . \quad . \quad . \quad 2$$

where γ_o = surface tension of water, γ_c = surface tension of the substance at concentration C; n and B are dimensionless constants; S and A are constants depending on the choice of concentration units, represented as S_p and A_p for percent concentrations or S_m and A_m for molar concentrations. Table 1 lists these constants for the representations of Freundlich and Szyszkowski. Detailed results are shown in Table 2, for which Szyszkowski's representation gives errors ranging from —0·28 p.c. to +0·57 p.c., whereas the range of errors of the Freundlich representation lies between —3·76 p.c. and +1·87 p.c. of the listed data. B and S_m increase and A_m decreases with the number of carbon atoms while 1/n has lower values for even numbers of carbon atoms than for the immediately preceding odd number. B, log A_m and log S_m show the greatest variations around 3–4 carbons in conformity wtih transitions in chemical properties in this region. On the heuristic considerations expressed in the preceding section, it is unlikely that acetamide could be replaced successfully by other amides. Should this be possible, however, the participation of II in the MR would be made very probable. Experimental investigation of this matter is in progress and will be reported in due time.

TABLE 1.

Freundlich and Szyszkowski constants of aliphatic amides at 18·5° C.

Amide	1/n	S_m	B	A_m
Formamide	0·78	0·019	0·10	1·78
Acetamide[a]	0·68	0·045	0·12	0·74
Propionamide	0·72	0·135	0·29	0·55
n-Butyramide	0·58	0·256	0·33	0·21
i-Butyramide	0·54	0·261	0·33	0·20
n-Valeramide	0·57	0·542	0·40	0·075
n-Caproamide	0·54	1·210	0·40	0·0165

a: Acetamide at 18° C. (Dunn and Polya, 1950).

The use of 1-phenyl-3, 5-dimethyl-1, 2, 4-triazole in MR.

Owing to the interesting biochemical properties of 1-phenyl-3, 5-dimethyl-1, 2, 4-triazole (Jackson and Polya, 1951) it was decided to investigate the possibility of its use in MR. For brevity, the term "triazole" will refer to this compound although other 1, 2, 4-triazoles have been and will be studied in our laboratory and other collaborating Institutes.

MR using acetamide and triazole respectively on identical cancer and normal sera are illustrated with Figs. 1 and 2 from which it will appear that the latter reagent offers the advantage of qualitative as well as quantitative differences. At present we have too little clinical material to assess the statistical significance of the value of triazole in the MR and we wish to report only on some fundamental features of the use of triazole.

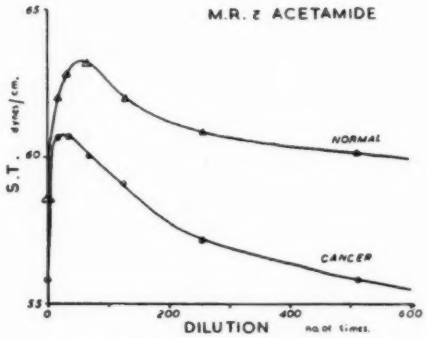

Fig. 1. Meiostagmin reactions of cancer and normal sera with 5 p.c. acetamide.

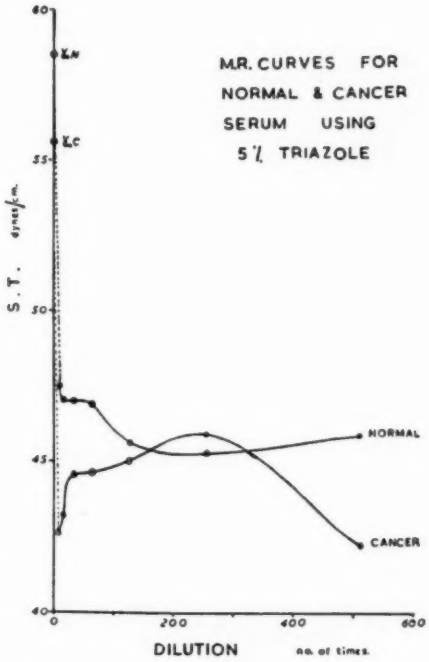

Fig. 2. Meiostagmin reactions of cancer and normal sera with 5 p.c. 1-phenyl-3, 5-dimethyl-1, 2, 4-triazole.

Attempts at producing MR curves similar to those obtained on cancer sera with artificial systems were made in order to obtain a preliminary idea of the nature of the reaction. Since all attempts to imitate cancer serum by pure and mixed albumin and globulin solutions for the purposes of the MR with acetamide have failed (Parkes, 1950), it will be sufficient to give a few illustrations only in Figs. 3 and 4 to show the nature of the results for comparison with Fig. 1. The variation of albumin : globulin ratios alone has little effect. The replacement of acetamide with Ringer solution ("Saline"), with practically the same surface tension as 5 p.c. acetamide, makes no substantial difference. This indicates that acetamide does not give the MR with albumin or globulin or albumin-globulin mixtures alone since the saline curves with either cancer or normal

sera are practically identical with the curves obtained on albumin or globulin solutions. A curve somewhat approximating cancer curves has been obtained by dissolving 5 p.c. albumin and 3 p.c. globulin in saline and adding to it the solvent free material extracted from an identical volume of cancer serum with ether. Under these conditions the typical maximum observed in the MR of normal and cancer sera appears but the G-value is distinctly negative. Also the maximum appears at a much higher dilution than in MR with normal or cancer sera.

TABLE 2.

Surface tensions (γ_c dyne/cm.) of aliphatic amides in water at 18·5° C.

Concentration p.c.	Amides					
	$HCONH_2$	$EtCONH_2$	α-$PrCONH_2$	β-$PrCONH_2$	α-$BuCONH_2$	α-$AmCONH_2$
0·05	..	..	..	..	..	70·4
0·10	..	..	..	..	..	68·0
0·20	..	..	..	..	..	64·1
0·25	..	..	..	..	69·7	..
0·30	..	..	..	..	..	61·1
0·40	..	..	..	..	..	58·7
0·50	..	71·7	70·7	70·2	66·9	56·7
0·6	..	..	..	..	..	54·8
0·75	..	..	..	..	64·2	..
0·80	..	..	..	..	..	52·0
1·00	..	70·9	68·5	67·7	62·5	49·8
1·25	72·5	..	..	..	61·0	..
1·5	..	..	..	..	59·5	..
2·0	..	69·2	65·4	64·5	57·0	..
2·5	72·1	..	..	..	54·5	..
3·0	..	67·8	62·9	62·2	52·5	..
4·0	..	66·7	60·7	60·2	49·7	..
5·0	71·5	65·4	59·5	58·7	47·0	..
6·0	..	64·4	57·9	57·0	..	..
7·5	70·9	..	..	..	..	..
8·0	..	62·8	55·2	54·5	..	..
10·0	70·4	61·7	53·5	52·8	..	..

Surface tension of water: 73·0 dyne/cm.

Acetamide, acetic acid and diacetimide: Dunn and Polya (1950).

Results of similar experiments on the MR with triazole in artificial systems are shown in Fig. 5. Comparison with Fig. 2 suggests some parallelism between cancer and globulin curves on one hand and normal and albumin curves on the other. Yet the curves for albumin-globulin mixtures are not obtained as a summation of the appropriate values of the albumin and globulin curves for each point. Clearly the process must be governed by the extent and nature of albumin-globulin interaction and the competition of "free" albumin and globulin for triazole. Qualitatively the results agree with the view that albumin forms relatively insoluble combinations with triazole at higher and globulin at lower concentrations. The normal meiostagmin curve may be explained by assuming

that most globulin is fixed or otherwise stabilized, leaving albumin only to react with triazole, whereas in cancer sera raised globulin levels, labilization of globulins or the occurrence of globulins which cannot be fixed or stabilized in the normal manner leads to predominant globulin-triazole interaction.

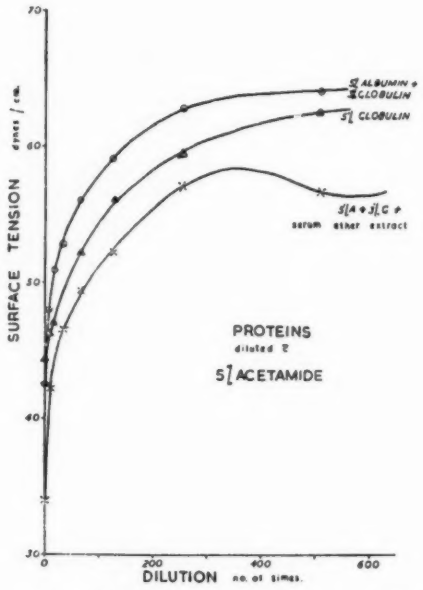

Fig. 3. Meiostagmin reactions of "artificial sera" with 5 p.c. acetamide.

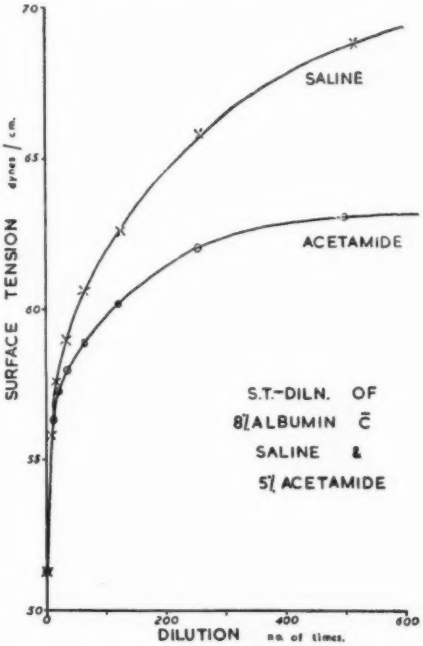

Fig. 4. Meiostagmin reactions of serum albumin with 5 p.c. acetamide and Ringer solution.

Although the concentration of nucleic acid in serum is negligibly low, the cytological effects of triazole suggested that some useful information may be gathered from the MR of thymonucleic acid. In order to avoid irregular dilution effects the behaviour of triazole and thymonucleic acid on dilution were studied. The Szyszkowski constants could not be determined accurately for the latter in a borate buffer of pH 7·89 as the slope of the curve is too low. The values for triazole are $B = 0{\cdot}25$, $A_P = 0{\cdot}13$ but only up to a concentration of 4–5 p.c., that is, the concentration used in our experiments: at higher concentration association increases rapidly. Thymonucleic acid as a 0·6 p.c. solution in the borate buffer was studied for its MR with 5 p.c. acetamide in the same solvent and a similar experiment was carried out with 0·8 p.c. thymonucleic acid and 5 p.c. triazole solutions. The results are shown in Fig. 6, from which

it is seen that neither of these MR resemble those of normal and cancer sera. The sharp minima, especially in the case of acetamide, suggest film penetration with complex formation (Cockbain and Schulman, 1939; Rideal, 1945). The minima occur at 4 dilutions (acetamide) and 16 dilutions (triazole). From these data, the concentrations stated above and the molecular weights of acetamide and triazole one finds that complex formation from 0·1 nucleic acid involves 0·056 M acetamide or 0·057 M triazole under the given experimental conditions.

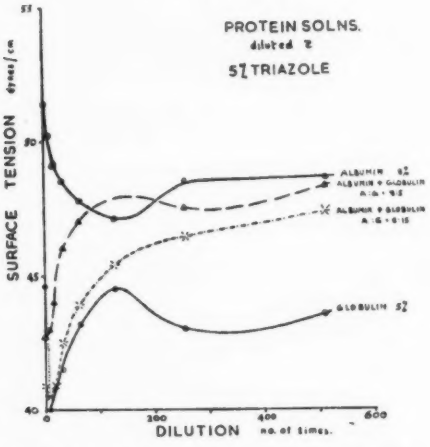

Fig. 5. Meiostagmin reactions of "artificial sera" with 5 p.c. 1-phenyl-3, 5-dimethyl-1, 2, 4-triazole.

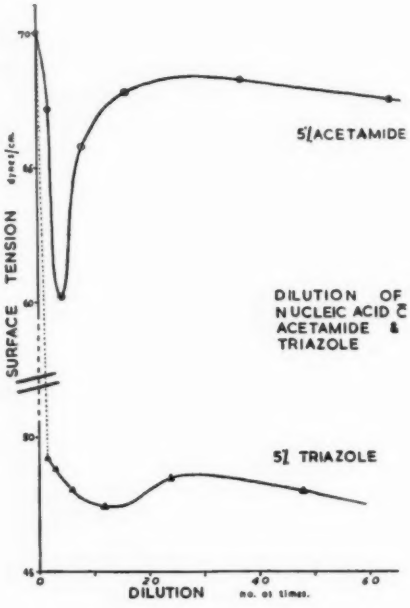

Fig. 6. Meiostagmin reactions of thymonucleic acid with 5 p.c. acetamide and 5 p.c. 1-phenyl-3, 5-dimethyl-1, 2, 4-triazole.

Experimental.

The technique of surface tension measurements and of MRs was the same as described before (Polya and Dunn, 1950). The curves shown in the Figures have been smoothed from 6-8 experiments. Figs. 1-5 have only qualitative value as illustrations of trends and some experimental points are not marked.

Formamide was dried and distilled, b.p. 114° C./18 mm. The solid amides were B.D.H. preparations except for *n*-caproamide, which has been prepared by A. B. Jack. All the solid amides were purified by repeated recrystallization from chloroform and petroleum ether (60–80°) to the following (corrected) melting points: acetamide 81° C.; propionamide 79·5–80° C.; *n*-butyramide 115° C.; isobutyramide 126–127° C.; *n*-valeramide 104° C.; *n*-caproamide 101–102° C.

The preparation of 1-phenyl-3, 5-dimethyl-1, 2, 4-triazole by Dr. A. A. Komzak and one of us (J.B.P.) will be described elsewhere.

SUMMARY.

New statistical data are presented for the meiostagmin reaction with acetamide and the reaction is discussed in relation to the structural modification of acetamide. Surface tension data are reported for other amides.

Some variations of the meiostagmin reaction are discussed, including meiostagmin reactions with 1-phenyl-3, 5-dimethyl-1, 2, 4-triazole and with acetamide in artificial systems. Both acetamide and 1-phenyl-3, 5-dimethyl-1, 2, 4-triazole combine with thymonucleic acid in the same stoichiometric proportions.

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